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Nitrogen Excretion in Developing Chick Embryos

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INTRODUCTION

OF the many examples of metabolic changes occurring during development, the most widely cited is that in developing chicks wherein the end product of nitrogen metabolism has been assumed to shift from ammonia to urea and finally to uric acid (Needham, 1931). This system appeared to be ideal for a proposed study of the mechanisms bringing about such changes in metabolism, providing the occurrence of these changes could be further substantiated. The experimental evidence upon which this pattern had been postulated consisted entirely of measurements of the total amounts of ammonia, urea, and uric acid in the allantois and the ratio of their total weights to the weight of the embryo. Nothing was reported concerning the changes in concentration of these substances nor their presence outside the allantois. Lack of this information makes it impossible to conclude that net synthesis occurs, particularly in the cases of ammonia and urea where the total amounts present are quite small.

If such a series of changes occurs in the excretion pattern of the developing chick, it is necessary that the enzymatic patterns of the organism undergo a concurrent series of transformations. In this connexion there appears in the literature only an incomplete analysis of arginase activity in developing chicks (Needham & Brachet, 1935).

In this paper there is presented an analysis of the nitrogenous contents of the allantois and the entire egg, a study of the fates of injected nitrogenous substances, and an analysis of embryos for the enzymes urease and arginase. These investigations have yielded evidence which we believe clearly shows that ammonia is not an excretion product and that the embryo does not pass through stages of ammonia or urea excretion characteristic of its evolutionary ancestry.

MATERIALS AND METHODS

Eggs used for these studies came from a flock of white leghorns and were incubated at 39° C. in a commercial incubator. Eggs were injected by placing

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0.5 ml. of either distilled water or the appropriate solution in the air-sac. Allantoic fluid was removed with a hypodermic syringe through a hole in the blunt end of the egg.

Chemical determinations

Ammonia was determined by the method of Boyce (1950) substituting Dow-Corning antifoam A for the antifoam agent used in the original procedure. This method involves the removal of ammonia from complex mixtures by passing air through an alkaline aliquot into 0.1 N H_2SO_4 which traps the volatile ammonia. The quantity present is then determined by the use of Nesaler's Reagent according to the method of Folin & Wu (1919). All analyses were run in duplicate.

Urea was determined by treating the sample with a standard solution of jack bean meal urease (Folin & Wu, 1919) and measuring the resulting ammonia by the method described above.

Uric acid in the allantoic fluid was determined by the glycerol-silicate method of Forsham *et al.* (1948).

Total nitrogen was determined by a modified Kjeldahl digestion (Ma & Zuazaga, 1942) followed by nesalerization according to the procedure used for ammonia determinations.

Enzyme determinations

Urease was measured by the method of Van Slyke & Cullen (1914) wherein a tissue homogenate is incubated with a standard buffered (pH 7.0) solution of urea, at room temperature for various time intervals. After stopping the enzymatic activity by addition of acid the amount of ammonia formed is determined as described above.

Arginase activity was determined according to the procedure of Van Slyke & Archibald (1946) as follows: (1) tissues are homogenized in an appropriate amount of distilled water; (2) 0.25 ml. of a 20 per cent. solution of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ is added to a 5 ml. aliquot of the homogenate; (3) the mixture is incubated for 20 minutes at 56°C . (to activate the enzyme); (4) after cooling, 2.5 ml. of an 18 per cent. solution of arginine (adjusted to pH 9.5) is added; (5) the reaction mixture is incubated at room temperature (approximately 25°C .); and (6) 1-ml. samples are removed after 0, 20, 40, and 60 minutes of incubation and placed in test-tubes maintained for 10 minutes at 90°C . in a water-bath to stop enzymatic activity. The 1-ml. samples are adjusted to pH 7 with 0.2 M phosphate buffer; the urea formed is hydrolysed with urease; the resulting ammonia is determined as described above.

RESULTS AND DISCUSSION

Ammonia excretion

Needham (1926a, 1931) has published data showing that the total amount of ammonia in the allantois increases during chick development from 3 μg . at 4 days to 56 at 13 days, and has shown that the ratio of allantoic ammonia to

weight of embryo decreases during this entire period. From these facts he postulated that chicks excrete ammonia in early stages and gradually lose this capacity with urea becoming the predominant excretory product.

TABLE 1

Ammonia in allantoic fluid and entire egg contents during development

Incubation time (days)	Concentration of NH_3 ($\mu\text{g. N/ml.}$)		Total NH_3 content ($\mu\text{g. N}$)	
	Allantois	Entire egg	Allantois	Entire egg
0	..	8 (3)†	..	350
1	..	11 (5)	..	500
2	..	10 (5)	..	450
3	..	12 (1)	..	550
4	..	11 (3)	..	500
5	8 (1)*	10 (2)	3	450
6	8 (5)	8 (2)	10	350
7	8 (4)	8 (3)	19	350
8	6 (3)	9 (3)	21	400
9	6 (3)	8 (3)	28	350
10	10 (1)	7 (4)	55	300
11	10 (5)	9 (1)	64	400
12	..	12 (2)	..	550
13	..	7 (2)	..	300

* Number of pooled samples analysed separately and values averaged.

† Number of eggs analysed separately and values averaged.

Our data are in agreement with his experimental findings that the total amount of ammonia in the allantois increases during development; however, measurements (Table 1) show that the concentration remains constant. The concentration of ammonia in the whole egg contents (homogenized) was found to equal that in the allantois, and the total amount in the developing egg remained relatively constant from the 1st through the 13th day (Table 1). Samples were pooled from a number of allantoic sacs (e.g. 13 on 5th day, 3 on 11th day) to obtain adequate volumes for analysis. The volumes of allantoic sacs were determined from a standard plot made by removing the fluid contents with a syringe from ten replicate eggs at each day and measuring the volume obtained. These values agreed very closely with those found by Fiske & Boyden (1926).

When 1 mg. of ammonia nitrogen (as a buffered ammonium sulphate solution) is placed in the air-sac of eggs which have been incubated for 6 days, it becomes equally distributed between the yolk and allantois raising the concentration of ammonia in each threefold. This excess ammonia disappears rapidly, the value for both yolk and allantois becoming equal to the controls injected with distilled water within 2 days (Table 2).

If the early chick embryo, from the 1st to the 5th day, is an ammonia-excreting organism, it is necessary that urease be present, otherwise purine nitrogen would

not be eliminated as ammonia. All attempts to demonstrate urease activity have been unsuccessful, even in the blastodiscs of unincubated eggs. The methods used were so sensitive that the production of 1 $\mu\text{g.}$ of ammonia nitrogen by an entire embryo in 1 hour would have been detected.

TABLE 2

Fate of ammonia injected as ammonium sulphate (1.0 mg. N) into 6-day-old developing eggs

Incubation time (days)	Concentration NH_3 ($\mu\text{g. N/ml.}$)			
	Allantois		Yolk	
	NH_3 injected	H_2O injected	NH_3 injected	H_2O injected
6	..	8 (5)*	..	7 (1)*
6.2	31 (2)*	11 (2)	29 (1)*	..
6.4	23 (1)	9 (1)	17 (1)	..
6.6	10 (5)	7 (2)	21 (4)	8 (1)
8	7 (2)	6 (3)	8 (3)	14 (1)
9	8 (4)	6 (3)	9 (2)	12 (1)
10	10 (3)	10 (1)	10 (1)	..
11	8 (7)	10 (5)	6 (1)	..

* Number of pooled samples analysed separately and values averaged.

These data show that (1) the *total amount* of ammonia in the allantois increases during development; (2) the *concentration* of ammonia in the allantois remains constant from the 5th through at least the 11th day; (3) throughout this period the concentration of ammonia outside the allantois equals that inside; (4) ammonia injected as ammonium sulphate into a developing egg rapidly becomes distributed uniformly in the yolk and allantois, increasing the concentrations in each to the same extent; (5) the excess ammonia resulting from the injection disappears from both the allantois and the yolk in less than 2 days' time; and (6) no urease activity is detectable from as early as the blastoderm stage.

The existing evidence does not warrant any conclusion concerning the presence of an active ammonia excreting mechanism during the first 5 days of development, and that after the 5th day there is no net excretion of ammonia into the allantoic sac.

Urea excretion

As in the case of ammonia, Needham (1926*b*, 1931) has reported data showing (1) that the total amount of urea in the allantois increases during chick development; (2) that the ratio of urea to the weight of the embryo increases from the 4th to the 8th day; and (3) that this ratio decreases from the 8th day to the time of hatching. He has postulated from these facts that the chick passes through a stage of high urea excretion.

Our analyses are in agreement that the total amount of urea in the allantois increases during development (Table 3). It was also found (1) that, unlike ammonia, the allantoic *concentration* of urea does not remain constant but increases from the 5th through at least the 11th day; (2) that the total amount of urea in the entire egg contents increases during the entire period studied; and (3) that the concentration of urea in the allantoic fluid is several fold higher than in the surrounding fluids (by the 11th day the allantois contains one-half of the urea present inside the entire egg, see Table 3).

TABLE 3

Urea in allantoic fluid and in entire egg contents during development

Incubation time (days)	Concentration of urea ($\mu\text{g. N/ml.}$)		Total urea content ($\mu\text{g. N}$)	
	Allantois	Entire egg	Allantois	Entire egg
0	..	2 (1)†	..	100
1	..	3 (1)	..	150
2	..	4 (3)	..	200
3	..	4 (1)	..	200
4	..	5 (3)	..	200
5	10 (1)*	3 (1)	2	150
6	11 (3)	..	14	..
7	16 (2)	8 (1)	37	350
8	21 (3)	6 (1)	69	250
9	26 (3)	6 (1)	112	250
10	29 (1)	12 (2)	154	550
11	31 (5)	9 (2)	198	400

* Number of pooled samples analysed separately and values averaged.

† Number of eggs analysed separately and values averaged.

TABLE 4

Fate of urea (1.0 mg. N) injected into 6-day-old developing eggs

Incubation time (days)	Concentration of urea in the allantois ($\mu\text{g. N/ml.}$)		Total amount of urea in the allantois ($\mu\text{g. N}$)	
	Urea injected	Water injected	Urea injected	Water injected
5	..	10 (1)*	..	2
6	..	11 (3)	..	14
6.2	50 (1)*	..	70	..
7	84 (1)	16 (2)	190	37
8	77 (1)	21 (3)	250	69
9	94 (1)	26 (3)	400	112
10	93 (1)	29 (1)	490	154
11	92 (1)	31 (5)	580	198

* Number of pooled samples analysed separately and values averaged.

When 1 mg. of urea nitrogen (as a urea solution) is placed in the air-sacs of eggs, which have been incubated for 6 days, it rapidly becomes concentrated in the allantoic sac and remains there (Table 4). By the 11th day 40 per cent. of all the injected urea has been concentrated in the allantois even though the sac occupies only 10 per cent. of the total volume.

If the developing chick embryo passes through a stage wherein urea is the primary excretion product and shifts to a stage wherein uric acid is the primary product, one should expect to find a shift in the relative amounts of each being excreted. The daily *increases in concentration* of urea in the allantoic fluid from the earliest time measured (5th day) through the 11th were approximately 1 (?), 5, 5, 5, 3, and 2 $\mu\text{g.}$ of urea N/ml. (see Table 3); comparable values for uric acid were 30, 36, 32, 46, 28, and 31 $\mu\text{g.}$ uric acid N/ml. (see Table 6). Hence, from the earliest times at which measurement could be made the relative *amounts* of each excreted were reasonably constant and uric acid is the major excretory product as early as the 5th day.

The presence of a ureotelic metabolism in a developing chick would require that the enzyme arginase be present in order for protein nitrogen to be eliminated. It has been shown (Needham & Brachet, 1935) that the arginase activity per milligramme moist weight of developing chick embryos decreases during development from the 3rd to the 15th day. If the arginase in the early chick embryo is the result of a recapitulation of a ureotelic excretory metabolism certain organs (primarily the liver) should possess a higher concentration of the enzyme than others (Hunter & Dauphinee, 1924).

TABLE 5
Arginase activity in developing chick embryos

<i>Incubation time (days)</i>	<i>Arginase activity*</i> ($\mu\text{g.}$ arginine hydrolysed/hour/mg. moist tissue)			
	<i>Entire embryo</i>	<i>Head of embryo</i>	<i>Body of embryo</i>	<i>Liver of embryo</i>
2.5	40.0	..	..	..
3.0	31.0	..	..	..
4.0	14.0	..	..	..
5.0	10.5	..	..	..
6.0	8.5	..	..	..
7.0	7.0	7.8	6.9	9.7
8.0	6.0	..	..	..
9.0	5.5	..	..	..
10.0	4.5	3.4	4.3	..
11.0	3.0	..	..	..
12.0	2.5	3.3	2.1	..
13.0	2.0	..	..	..
14.0	1.5	1.8	1.9	..
15.0	1.0	0.9	1.0	..

* Each value reported is the average of at least two separate determinations on pooled samples from several embryos.

We have measured the arginase activity of intact embryos from 2.5 to 15 days, of separated heads and bodies from the 7th to the 15th day, and of isolated livers of 7-day-old chick embryos. No differences in activity were found (Table 5). On the basis of these data it is concluded that during the mesonephric stage of development chicks do not recapitulate the arginase distribution associated with a typical ureotelic physiology.

These studies show: (1) that the total amount of urea in the allantois increases during development; (2) that the concentration of urea in the allantoic sac also increases from the 5th through at least the 11th day; (3) that the concentration of urea is greater inside the allantois than outside; (4) that the injected urea becomes concentrated in the allantois; (5) the fraction of nitrogen excreted as urea remains relatively constant from the 6th through at least the 11th day; and (6) that arginase is not distributed among the various tissues of the developing chick in a manner characteristic of ureotelic organisms.

It is concluded that the developing chick excretes urea, but that the rate of urea excretion does not reach a maximum at any particular stage, and that the organism does not recapitulate a typical ureotelic excretion physiology.

Uric acid excretion

Although uric acid excretion has been extensively studied by many investigators (a list has been compiled by Needham, 1931), it was included in our studies for purposes of obtaining a more complete picture (Table 6).

TABLE 6

Fate of uric acid (0.5 mg. N) injected into 6-day-old developing eggs

Incubation time (days)	Concentration of uric acid in the allantois ($\mu\text{g. N/ml.}$)		Total amount of uric acid in the allantois ($\mu\text{g. N}$)	
	Uric acid injected	Water injected	Uric acid injected	Water injected
5	..	7 (1)*	..	21
6	..	37 (4)	..	48
6.2	74 (4)*	45 (4)	104	65
6.4	81 (2)	52 (2)	130	86
6.6	99 (2)	54 (4)	180	105
7	90 (5)	73 (5)	210	168
8	117 (2)	105 (2)	390	340
9	185 (2)	151 (2)	790	650
10	247 (2)	179 (3)	1,300	950
11	295 (1)	210 (1)	1,860	1,320

* Number of pooled samples analysed separately and values averaged.

When half a milligramme of uric acid nitrogen (as a sodium ureate solution adjusted to pH 7) was placed in the air-sac of eggs which have been incubated

for 6 days, it gradually accumulated in the allantoic sac until essentially all of it appeared there by the 11th day (Table 6).

By dividing the amount of uric acid by the weight of the embryo, Needham (1926*b*, 1931) obtained an excretion curve which is essentially zero from the 4th to the 7th day, which raises to a peak at the 11th day and then decreases. This curve can be interpreted as meaning that the embryo changes from a urea-excreting organism to an essentially uric-acid-excreting organism during the period from the 7th to the 11th day. If the change were as dramatic as would appear from this particular method of analysis of the uric-acid data, one should expect to find a shift in the relative amounts of each being excreted. As was shown previously (section on urea excretion) the daily increments in concentration in the allantois due to urea and uric acid are essentially constant from the 5th through at least the 11th day, and throughout this entire period the value for urea is only about one-sixth that for uric acid. Hence, from the earliest times at which measurements could be made the relative *amounts* of each excreted were reasonably constant and it is concluded that uric acid is the major excretory product as early as the 5th day.

Nitrogenous composition of allantoic fluid in developing chicks

To conclude this study of nitrogen excretion in developing chicks the total nitrogen in the allantois was determined to find out how much of the nitrogen present was accounted for by ammonia, urea, and uric acid.

Our analyses leave about 60 per cent. of the nitrogen to be accounted for. This is considerably more than calculated by Needham (1931) from data compiled from several investigators. Our unassignable fraction is larger than his value primarily because our uric-acid values were lower than those he used (Fiske & Boyden, 1926); however, our values are actually higher than Needham's (1926) own experimental values.

TABLE 7

Nitrogenous contents of the allantoic sacs of developing chicks

Incubation time (days)	Total nitrogen (μ g. N/sac)	Per cent. of total nitrogen		
		NH ₃	Urea	Uric acid
5	93	3.2	2.2	2.2
6	325	3.1	4.3	17.5
7	620	3.1	6.0	29.0
8	1,060	2.0	6.5	32.1
9	1,980	1.4	5.7	32.8
10	2,900	1.9	5.3	32.4
11	3,700	1.7	5.4	35.7

CONCLUSIONS

Ammonia excretion

Because (1) the concentration of ammonia inside and outside the allantois are constant and equal from the 5th through at least the 11th day; (2) ammonia injected into the egg moves rapidly and equally into the yolk and allantois (rapidly disappearing from both); and (3) no urease activity was detected, it is concluded that ammonia is not an excretion product after the 5th day of development, that it need not necessarily be considered a product of active excretion prior to this time, and that the increase in total ammonia in the allantois is due only to the increasing volume of that organ.

Urea excretion

Because (1) the concentration of urea inside the allantois is greater than that outside and increases during development; (2) urea injected into the egg moves rapidly into the allantois and remains there; (3) the increments of urea concentration in the allantois are essentially constant from the 5th to the 11th day; and (4) arginase is uniformly distributed among the various tissues of the embryo, it is concluded that although urea is an excretion product from the 5th through at least the 11th day, no predominantly ureotelic stage exists during chick development.

Uric acid excretion

Because (1) the concentration and total amount of uric acid in the allantois increases from the 5th to at least the 11th day; (2) uric acid injected into the egg accumulates in the allantois; and (3) the daily increments of uric-acid concentration in the allantois are equal from the 5th through at least the 11th day, it is concluded that uric acid is a major excretion product from the 6th to the 11th day and that no marked shift from urea to uric-acid excretion occurs during this period.

Total nitrogen in the allantois

The total amount of nitrogen was determined and 40 per cent. was accounted for as ammonia, urea, or uric acid.

SUMMARY

1. An analysis, more extensive than hitherto, has been made of nitrogen excretion in developing chicks. It was found that the concentration of ammonia in the allantoic fluid was constant from the 5th to the 11th day and equalled the concentration in the extra-allantoic fluids, e.g. yolk. The increase into total ammonia content of the allantois during development is apparently due to the increase in volume of the organ and not to excretion by the embryo.

2. The concentration of urea in the allantoic fluid increases during development and injected urea concentrates in that organ.

3. The total amount and concentration of uric acid in allantoic fluid increases during development. The ratio of urea nitrogen to uric-acid nitrogen is relatively constant from the 6th to the 11th day of development indicating that no major shift from urea excretion to uric-acid excretion occurs.

4. No urease activity was observed even in the blastodiscs of unincubated eggs.

5. The distribution of arginase activity in 7-day-old chicks did not resemble that of ureotelic organisms. Activity of heads, bodies, and livers were essentially the same.

6. From these results it was concluded that ammonia is not an excretion product and that the developing chick does not pass through either a typical ammonotelic or ureotelic stage of excretion.

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(Manuscript received 26 : vii : 56)

The Effect of Steroid Hormones on the Brown Trout (*Salmo trutta* L.) during the Period of Gonadal Differentiation

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WITH TWO PLATES

INTRODUCTION

IN tetrapods (see Witschi, 1950) and in elasmobranch (Chieffi, 1949) it appears that the gonadal primordium is derived from three sources, the primary germ-cells which migrate from the endoblast, the cortex formed from the somatopleure in the region below the Wolffian duct, and the medulla, which Vannini (1950), Vannini & Sabbadin (1954), and Chieffi (1952) claim to have a common origin with the interrenal, from the ventral mesoderm at the junction of the somatopleure and the splanchnopleure. The sex of the gonads which develop depends on the relative activity of the cortex and medulla in producing cortecins and medullarins, the cortex being the more active in females whilst in the males the medulla is dominant. Secretions of the gonads then determine which type of gonoduct shall develop and, at a later stage, the appearance of accessory sexual organs and secondary sexual characters. It is generally agreed that the inductions subsequent to the differentiation of the gonads are produced by steroid hormones, but Witschi has maintained that the sexual inductors have large molecules, are not chemically related to the sex hormones, and are probably proteins. Other authors on the contrary claim that these sexual inductors also are closely related to the sex hormones (for evidence see Dantchakoff, Gallien, Padoa, Vannini, Witschi, Wolff *et al.*, in *Colloque* (1950)). It is certainly true that the gonadal and suprarenal hormones of the adult often have powerful inductor-mimetic properties. These have been demonstrated particularly clearly in the Anura and in birds. Mammals were thought to be refractory, but Burns (1955) has shown oestradiol to have a cortecin-like action in *Didelphys*.

The reproductive organs of teleosts and holosteans differ fundamentally from those of other gnathostomes, since not only do the gonoducts, where present,

¹ This work was commenced in the Department of Zoology, University of Sheffield, continued at the Instituto di Zoologia, Università di Padova, Italy, and concluded in the Department of Zoology of the Durham Colleges in the University of Durham.

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TABLE 1
Effect of steroid hormones on the juvenile gonads in teleosts

Authority	Organism	Substance	Method of administration	Effect
Castelnuovo (1937)	<i>Cyprinus carpio</i>	oestrone testosterone }	injected	both → spermatogenesis only differentiated ♂s studied
Padoa (1937, 1939a)	<i>Salmo irideus</i>	oestrone*	dissolved in medium	→ ♀ in many ♂s, inhibition if treatment intense
Padoa (1939b)	<i>Hepatus hepatus</i> (an hermaphrodite)	oestrone*	dissolved in medium	spermatogones → spermatocytes
Régnier (1938, 1939)	<i>Xiphophorus helleri</i> <i>Lebistes reticulatus</i>	testosterone " }	fed, injected, or as oily emulsion in medium	→ ♂ or inhibition
Baldwin & Goldin (1939)	<i>Xiphophorus</i>	testosterone	injected	→ ♂ in ½ of ♀s†
Eversole (1941)	<i>Lebistes</i>	testosterone pregneninolone }	injected	both → spermatogenesis in ♂s and inhibition in ♀s
Berkowitz (1941)	<i>Lebistes</i>	oestrol oestradiol stilboestrol }	fed	moderate doses → ♀ high doses or long treatment → castration
Lepori (1945)	<i>Gambusia holbrooki</i>	oestrone testosterone }	fed or injected	accelerated development in both sexes (spermatogenesis in ♂s spermatogenesis and precocious oogenesis in young ♀s)
Yamamoto (1953)	<i>Oryzias latipes</i>	oestrone stilboestrol }	fed fed	→ ♀

→ ♀ } indicate induction of sexual inversion.
 → ♂ }

* Extract used caused masculinization in frog tadpoles.

† Witschi & Crown (see Witschi, 1939) did similar experiments with similar results. About half of female *Xiphophorus* are liable to spontaneous sexual inversion.

develop largely from the gonads, but also according to D'Ancona (1943, 1950, 1955), there is no medullary contribution to the gonad. Experiments with sterols on juvenile teleosts have been largely restricted to cyprinodonts, and the results have been less easy to interpret than those obtained in amphibia and birds. They are summarized in Table 1. It was decided to investigate the effects of four major groups of steroid hormones, oestrogens, androgens, progestins, and corticoids, on an unspecialized teleost. *Salmo trutta* was used, as fertilized eggs are easily obtained, the young stages are a convenient size for histological and experimental purposes, and since Padoa had already used *S. irideus* for similar work. Subsequent study has somewhat modified the conclusions reached in a preliminary account (Ashby, 1952) of this and other work on *S. trutta*.

MATERIALS AND METHODS

Alevins were reared in static water at 5° C. and shortly before the experiments were started, 170 days after fertilization, the temperature was raised to 8° C., which Brown (1946) found excellent for trout in the laboratory. The alevins were fed daily on chopped liver, with plankton as supplement, and the cold room was lit twelve hours a day. Six experimental cultures of 50 alevins each were set up in 5-litre rectangular glass tanks. Two hundred and fifty alevins divided between

TABLE 2
Treatments given to experimental cultures

Culture	Concentration of hormone	Maximum concentration of alcohol reached
(a) Alcohol-treated controls	..	0.5 c.c./litre*
(b) Oestradiol, low concentration	50 γ /litre	0.05 "
(c) Oestradiol, high concentration	300 γ /litre	0.3 "
(d) Testosterone	50 γ /litre, raised to 60 γ after 18 days	0.06 "
(e) Progesterone	50 γ /litre, raised to 100 γ during experiment†	0.1 "
(f) DOCA	100 γ /litre, raised to 400 γ during experiment‡	0.2 "

* In the preliminary report (Ashby, 1952), this figure was wrongly given as 0.25 per cent.

† The concentration of progesterone was raised to 60 γ /l. 18 days, and to 100 γ /l. 52 days after the experiments started.

‡ The concentration of DOCA was raised to 120 γ /l. after 18 days, to 200 γ /l. after 52 days, and to 400 γ /l. gradually between the 87th and 95th day of the experiments.

three 25-litre tanks acted as untreated controls. Unesterified oestradiol, testosterone, and progesterone (Schering) were made up as 0.1 per cent. alcoholic solutions and desoxycorticosterone acetate (DOCA) (Ciba) as a 0.2 per cent. solution, the hormones not being sufficiently soluble to make up in water. Fresh hormone was added daily when the water was changed. Foote (1941) had found that oestrogens were stable in water for several days but Witschi (personal communication) reports that androgens may disappear through bacterial destruction, and should be replenished daily. The initial dosages of progesterone and DOCA,

being near their lower limit of effectiveness in tadpoles, were raised when it had become obvious that the hormones were not poisoning the alevins. The treatments are summarized in Table 2.

The alevins remained lively and voracious in all the cultures except those treated with oestradiol, where growth was much retarded and casualties were heavy. The growth-rate was also slowed in the culture treated with testosterone. Samples of the untreated controls were fixed in Zenker at intervals up to 300 days after fertilization and 130 days after the experiments were started. Most of the experimental cultures were fixed three weeks before the last untreated controls. That treated with the higher concentration of oestradiol was fixed 40 days previously, because there were only ten survivors, all of which had almost ceased to feed. The culture treated with progesterone was also fixed early because the young trout of this culture were growing more quickly than those of any other, and it was suspected at the time, from observations on *Anguilla*, that the degree of sexual development would depend on body-length rather than age. A third of this culture had been accidentally killed a month after the experiments started by accumulation of excretory products due to failure to change the culture medium one weekend. The individuals concerned were fixed soon after death and proved to be suitable for histological study.

TABLE 3
Details of individuals sectioned

<i>Culture</i>	<i>Age fixed</i>	<i>Length of treatment</i>	<i>Number sectioned</i>	<i>Average body-length in culture or sample</i>
	(days)	(days)		(mm.)
Untreated controls	169	..	5	26
	192	..	4	27½
	206	..	4	28
	232	..	5	29
	267	..	13	34½
	300	..	21	39
Alcohol-treated controls	281	111	28	34
Oestradiol-50 γ/l.	281	111	13	29
Oestradiol-300 γ/l.	240	70	10	27½
Testosterone	281	111	29	29½
Progesterone	200	30	14	..
	254	84	16	33
DOCA	282	112	20	36

The fixed material was impregnated with Steedman's ester wax, and after removal of the head, tail, and dorsal musculature, was double embedded in paraffin wax of 54° C. melting point. The region between the pronephros and the anus was serially sectioned, usually at $7\frac{1}{2}$ μ, stained with Heidenhain's iron haematoxylin or azan, and mounted in balsam. Details of the animals sectioned are given in Table 3.

RESULTS

1. *Development of the reproductive system in the untreated controls*

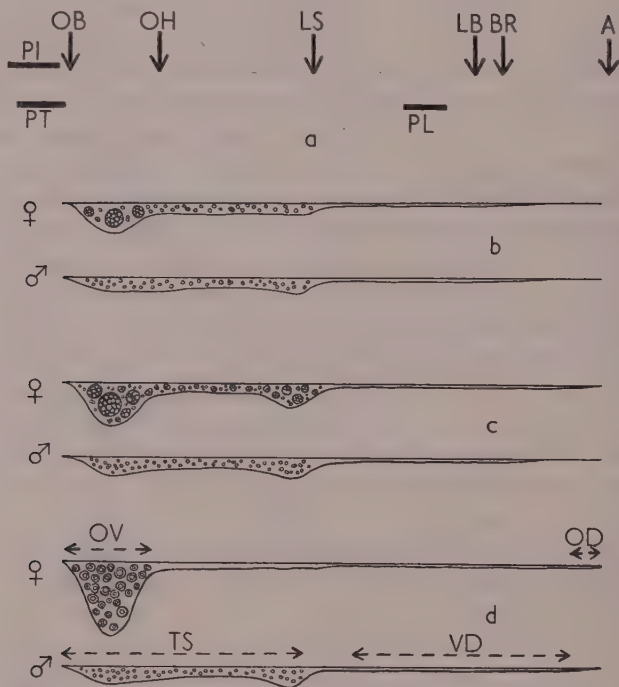
At the beginning of the experiments (169 days after fertilization), whereas most organs were fully differentiated and yolk absorption was nearly complete, the gonads were in an early stage of development; they consisted of folds of somatopleure running the length of the body-cavity containing protogones of about $14\ \mu$ diameter in the anterior two-thirds of their length. Some of their nuclei had entered the leptotene stage of meiosis, but with a few exceptions the cell volume had not increased. The only feature distinguishing the sexes was the presence in the female of a swelling at the anterior end of each gonadal fold containing large nests of up to 60 protogones and a few capillaries (Text-fig. 1b; Plate 1, figs. A and C). Elsewhere protogones occurred singly or in pairs, and no vascular tissue was present. The epithelial cells on the lateral aspect of the swollen region of the female gonad were slightly enlarged and crowded together in comparison with the epithelium elsewhere on the gonadal folds.

Development took place much more rapidly in the *females*, the swollen region of the gonadal folds becoming the definitive ovary. A month after the experiments commenced (192 and 206 days after fertilization) several oocytes had reached diplotene, and after two months (age 232 days) had entered the second growth phase, being $40\ \mu$ in diameter in the most advanced female but still remaining members of nests (Text-fig. 1c). In this female, deep pits had developed on the germinal side of the definitive ovaries which were the precursors of ovarian grooves. The protogones behind the definitive ovary sometimes divided to form nests during the first month, but after two months most of these cells had disappeared and many of those remaining showed signs of degeneration. Below the bladder the folds were attached to the mesentery instead of to the dorsal part of the coelom. Anteriorly the distinction between the lateral and medial epithelium remained slight, but posteriorly it became marked, some of the cells of the lateral epithelium adjoining the suspensory ligament being as large as $14\ \mu$ deep and $12\ \mu$ across. They had clear cytoplasm, and some were ciliated. This hypertrophy extended on to the peritoneum of the body-wall situated immediately laterally to the gonadal folds.

Three months after the experiments started (267 days after fertilization), the pits on the lateral aspect of the gonads had changed into deep and wide longitudinal grooves. At the base of these grooves the peritoneal cells of the germinal epithelium were much enlarged and were closely associated with concentrations of protogones and small oocytes. The largest oocytes ranged from 45 to $65\ \mu$ in diameter in the various females.

Four months after the experiments started (300 days after fertilization and three weeks after most of the experimental cultures were fixed) the ovaries had grown considerably (Text-fig. 1d) and the number of grooves on the germinal side had increased, sometimes several being seen in a transverse section. Most

of them were deep and narrow, and some were closed over at least in places (Plate 1, fig. B). The size of the larger oocytes had increased to between 60 and 75 μ in diameter. The degree of differentiation of the gonads continued to be correlated with the age of the individual rather than with its length, which varied in this sample from 31 to 49 mm.



TEXT-FIG. 1. Diagrammatic representation of gonadal development of untreated controls, in side view. *a*, The main levels in nearby organs used for reference. *b*, Gonadal folds at start of experiments. *c*, Gonadal folds one month later. *d*, The gonads at conclusion of experiments. A, anus; BR, anterior limit of bladder; LB, posterior limit of swim-bladder; LS, posterior limit of loop of stomach; PI, pronephros and interrenal (embedded in lymphatic tissue); PL, pelvic fin attachment; PT, pectoral fin attachment; OB, opening of swim-bladder into oesophagus; OD, median terminal oviduct; OH, opening of hepatic duct into duodenum; ov, ovary; TS, testis; VD, vas deferens.

There was also a great increase in the number of small oocytes associated with the hypertrophied epithelium lining the ovarian grooves. All the cells of the latter were indistinct in outline and varied in size and shape, their nuclei ranging from 3 $\mu \times 4 \mu$ to 9 $\mu \times 11 \mu$ in size. Those immediately adjacent to the coelomic fluid stained very faintly and were sometimes practically invisible. This epithelium

was associated with the production of follicle and stroma cells as well as oogonia, and will alone be referred to as the germinal epithelium. It was clearly distinct in appearance from the areas of hypertrophied cells on the lateral aspect of the suspensory ligament. Oogenesis followed the description by D'Ancona (1943) for *Anguilla*. The number of stroma cells was fairly small. The vascular supply was large in relation to the amount of stroma but small in comparison with the total volume of the ovaries.

Posteriorly, all protogones had disappeared, and the previously sharp distinction between the epithelium on the median and lateral aspect of the gonadal folds was much less prominent. The gonadal folds extended in most cases to the posterior limit of the coelom as delicate structures observed only with difficulty. Below the middle and posterior part of the bladder the gonadal folds were attached to the mesentery immediately above the rectum, and that part of the mesentery lying dorsal to them had disappeared. Below the posterior section of the bladder, the folds had grown dorso-laterally to fuse with the coelomic epithelium of the body-wall, thus forming a single median oviduct. In some instances it continued a short distance beyond the posterior limit of the coelom.

In the *males* the number of protogones continued to increase slowly particularly anteriorly and posteriorly, during the first two months of the experiments (up to the age of 232 days), but they remained the only type of germinal cell present (Text-fig. 1c). A more obvious change was the increase in the amount of both vascular and connective tissues, particularly at and immediately behind the posterior limit of the protogones. These tissues became concentrated mainly below the medial, i.e. the 'vascular' epithelium and at the hilum of the gonads, which resulted in the testes becoming triangular in cross-section, a feature which later became more pronounced and one which Padoa regards as a diagnostic masculine feature of juvenile *Salmo*. Early indications of a developing vas deferens were seen near the pelvic level in one case after a month, and clearly defined vasa deferentia after two months.

TABLE 4
Sex ratio in the control and experimental cultures

Culture	Indeter- minable	Female	Male
Untreated controls	..	23	29
Alcohol-treated controls	..	15	13
Oestradiol 50 γ /l.	3	4	6
Oestradiol 300 γ /l.	..	7	3
Testosterone	7	12	10
Progesterone fixed at:			
200 days	2 } 3	4 } 15	8 } 12
254 days	1 }	11 }	4 }
DOCA	3	14	3

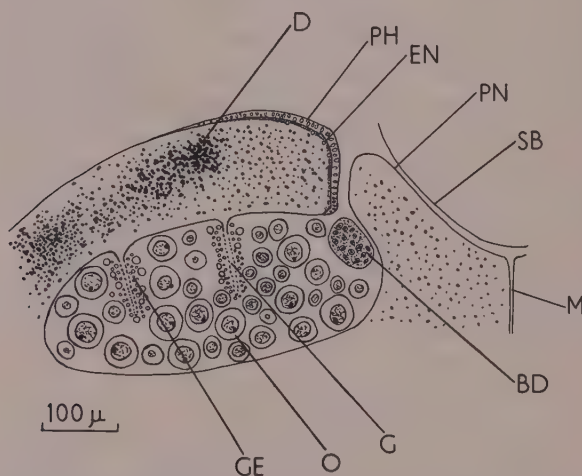
There had been little further change a month later (age 267 days), but by the age of 300 days, about four months after the experiments started, the orientation

of the germinal cells to form strings of cells was being caused by continued increase in amount and by rearrangement of stroma and connective tissue, there having been little or no change in the number of protogones (Plate 1, fig. D). One individual possessed anteriorly a few oocytes in the second phase of growth, but otherwise its gonads resembled those of other males fairly closely. At the age of 267 days at least traces of vasa deferentia were present in most of those examined, and at 300 days they were seen in all but one instance (Text-fig. 1d and Plate 1, fig. E). They were still confined to the posterior sterile region of the gonadal folds. At their hind end they were attached to the mesentery, like the gonadal folds of the older females. There was a normal sex ratio in the untreated controls as a whole (see Table 4).

II. Modifications (except of interrenal) induced in the experimental cultures

(a) Control culture treated with alcohol

The sex ratio was normal as in the untreated controls, and the reproductive system was in general in a similar condition to that in untreated animals aged 267 and 300 days, but there were some peculiarities. There was an increase in



TEXT-FIG. 2. Diagram of ovary in transverse section in the controls treated with alcohol. BD, large blood-vessel with chromophil deposit in plasma; D, chromophil material in coelom; EN, extruded nucleus; G, groove on lateral aspect of ovary; GE, germinal epithelium; M, mesentery; PH, peritoneum liable to hypertrophy and ciliation; PN, peritoneum never hypertrophied; O, oocyte in second growth phase; SB, swim-bladder.

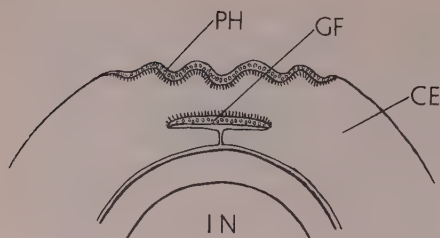
the vascular supply to the reproductive system and in the tendency more particularly in females, for the peritoneal cells covering the dorso-lateral aspect of the gonad, the lateral side of the suspensory ligament, and the adjacent part of the body-wall to be enlarged and ciliated (Text-fig. 2 and Plate 1, fig. F). In some females the amount of germinal tissue was reduced. In most individuals there

was an increased affinity for basic stain, of material in the body-fluids. This was usually in droplet form, whereas the lightly staining precipitates of untreated animals were fibrous in nature. The amount was considerable in 3 of the 15 females and 5 of the 13 males. The quantity seen in the coelom and in the blood and lymph systems seemed to be correlated. It often accumulated to a marked extent in the connective tissue surrounding the swim-bladder. Within the gonads, the droplets looked like spermatozoa, but this appeared to be only a coincidence as the size of individual granules or droplets was very variable.

In *females* the right ovary was reduced more in size than the left in 13 out of the 15 cases. The reduction was in the system of grooves and cavities and the number of small oocytes, the size and number of large oocytes appearing normal except in one female where few oocytes of any type were present, and in another where the largest oocytes were only $40\ \mu$ in diameter. Generally the oocytes ranged from 50 to $70\ \mu$ in diameter.

Peritoneal cells on the lateral side of and immediately lateral to the suspensory ligament of the ovary when slightly affected by hypertrophy, were squamous or cubical, and had invisible boundaries where they bordered other cells, lightly staining structureless cytoplasm, and nuclei of about $3\ \mu \times 5\ \mu$ in size with a prominent nuclear membrane and single nucleolus. Strongly affected epithelium was columnar, ciliated, and in places two cells deep, individual cells even reaching $6\ \mu$ across and $20\ \mu$ deep, with cilia $7\ \mu$ long and nuclei up to $5\ \mu \times 9\ \mu$ in size. The nucleoli remained well defined, but the nuclear membrane had lost its prominence and there was a large-meshed chromatin network. Some of the cells projected into the coelom and appeared to be breaking up, leaving their nuclei exposed to the coelomic fluid. Thus although few mitoses were seen, it appeared that cell replacement was rapid. A few detached nuclei apparently derived from this epithelium were sometimes seen in parts of the coelom sheltered from movements of the coelomic fluid. Peritoneal hypertrophy was marked in 6 of the 15 females and slight or very slight in 4.

Behind the fertile regions the gonads were merely folds as in the older untreated females, but the epithelial cells on their lateral aspect were to a variable extent enlarged, crowded, and ciliated and the core of connective tissue contained large blood-vessels. Towards the level of the pelvic fins, in individuals where hypertrophy was marked, it spread laterally and ventrally to a little below the mid-lateral line, but the epithelium had not become columnar except in the immediate vicinity of the gonadal folds. Behind the pelvic fins the area of hypertrophy was restricted to the immediate neighbourhood of the gonadal folds, and was reduced in intensity. In individuals where the folds extended a long way posteriorly, the changes in their attachment below the bladder, already described in the untreated controls, were made easier to observe by the enlargement of the peritoneal cells covering them, the hypertrophy continuing to the posterior limit of the coelom (Text-fig. 3), and the terminal median oviduct when present being lined by a columnar epithelium (Plate 1, fig. G).



TEXT-FIG. 3. Diagram showing distribution of hypertrophied peritoneum in transverse section, immediately anterior to the median oviduct in females strongly affected by treatment with alcohol. CE, coelom; GF, fused gonadal folds bearing hypertrophied epithelium; IN, intestine; PH, hypertrophied and ciliated somatopleure.

Abnormalities in the *males* were less apparent, the appearance and quantity of germinal tissue differing little from normal. Hypertrophy of the peritoneum in the lateral angle of the gonad was absent or very slight in 7 and marked in only 2 of the 13 males. The blood-supply to the tissues associated with the reproductive system was very great, and the development of the vasa deferentia had also been much accelerated, these being considerably larger than in the untreated controls (Plate 1, fig. H) and extending forward to the posterior limit of the germinal tissue, where the dimensions in transverse section reached $80 \mu \times 110 \mu$ in one instance. Sometimes there was a network of smaller tubules at this level instead of there being one large vas deferens. The epithelium of the lateral aspect of the left testis in three of the males was slightly grooved anteriorly in a similar manner to the ovaries although to a much lesser extent.

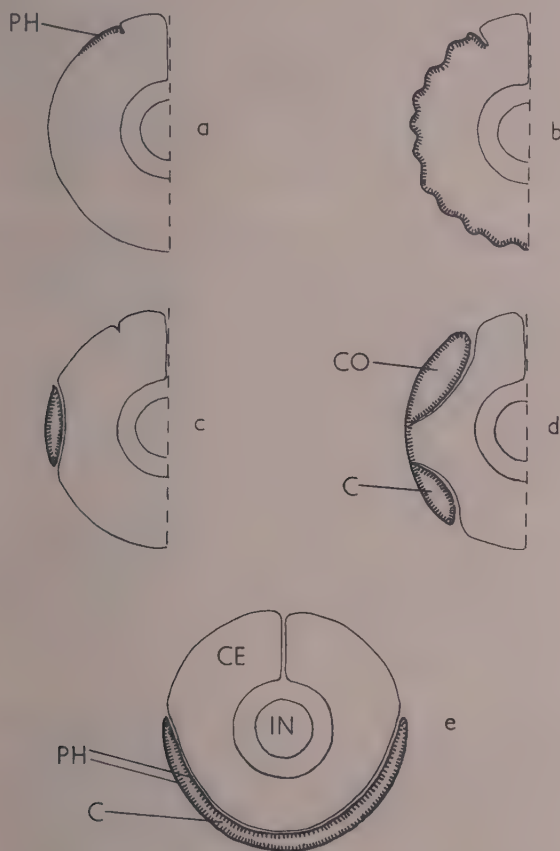
(b) *Culture treated with 50 γ /l. of oestradiol*

The treatment had a powerful inhibitory effect on the germinal tissue (almost causing the elimination of the gonads in some cases), and a stimulatory effect on the growth of the peritoneum, a large area of which in all individuals developed into a columnar epithelium, similar in structure to that seen in some of the controls treated with alcohol (Plate 2, fig. K). The tissues associated with gonadal areas anterior to the pelvic level had an enormous vascular supply, and the body-fluids contained a large amount of deeply chromophil material. In all members of the group the gall-bladder was in a collapsed condition, and what remained of its cavity together with that of the bile-ducts contained a large quantity of a similar deposit. The tubules of the pronephros were much enlarged.

A few oocytes in the second phase of growth were present in 4 out of the 13 animals fixed, their maximum diameter in the individuals concerned varying from 25 to 45 μ (Plate 1, fig. J). They were generally to some extent deficient in follicle cells. Stroma cells were few in number or absent. No member of the group entirely lacked germ-cells, but often there were only a few widely scattered groups of protogones or cells in meiotic prophase. Protogones were difficult to identify since their follicles, if present, were abnormal. Some of the small germ-cells were definitely degenerating and resembled the necrotic protogones seen posteriorly to the definitive ovaries in older females.

Vasa deferentia were present in 6 members of the group. They were most

easily seen at the level of the anterior part of the bladder, but they remained distinct from the hypertrophied peritoneum as far forward as the posterior limit of the stomach loop, where they were lost in complex peritoneal folding. Vasa



TEXT-FIG. 4. Diagram showing distribution of hypertrophied peritoneum in transverse section in the cultures treated with oestradiol and testosterone. *a*, anteriorly; *b*, at pelvic level; *c*, posteriorly; *d*, where lateral cavities developed; *e*, where large ventral cavity formed. *c*, cavity; *CE*, coelom; *CO*, cavity resembling oviduct; *IN*, intestine; *PH*, hypertrophied and ciliated epithelium.

deferentia and oocytes did not occur together in the same animal. It thus seems reasonable to assume that the six individuals in which vasa deferentia were present were genotypic males, and that the four possessing oocytes in the second phase of growth were females. Two of the remainder definitely possessed neither oocytes nor vasa deferentia.

The hypertrophy of coelomic epithelium in the anterior third of the body-

cavity corresponded approximately to that in the most strongly affected of the controls treated with alcohol, but the area affected was greater and the hypertrophy was more intense (Text-fig. 4a). About the level of the posterior limit of the stomach loop, the hypertrophy became more extensive and spread round the body-cavity at least to the mid-lateral and often to the mid-ventral line (Text-fig. 4b and Plate 2, fig. L) but the peritoneum of the gut and mesentery and that situated between the mesentery and the gonadal areas was never affected. Behind the level of the pelvic fins the intensity of the hypertrophy diminished ventrally and dorsally, so that further back only the peritoneum situated laterally was affected (Text-fig. 4c). It ceased altogether at the level of the anterior limit of the bladder.

Where strongly hypertrophied the epithelium was $30\ \mu$ deep and contained cells up to $25\ \mu$ deep which sometimes had a very well-defined boundary at the surface of the epithelium. The peritoneum increased in area as well as in depth, which resulted in it becoming corrugated or folded over on itself. When the latter occurred at the dorsal limit of hypertrophy, the resulting structures sometimes resembled big sterile gonadal folds. Often the edge of one of these folds fused with another part of the peritoneum with which it came in contact, thus causing the enclosure of a coelomic channel. When formed dorsally such channels in section resembled large oviducts (Text-fig. 4d). The formation of channels was most liable to occur behind the pelvic level, where sometimes two or three were present on each side of the body-cavity. In one or two instances folds which were dorsal in origin met in the mid-ventral line, thus giving all but the dorsal side of the coelom a double wall (Text-fig. 4e).

(c) Culture treated with 300 γ /l. of oestradiol

The survivors fell into two groups of 7 and 3 resembling females and males respectively. In the former, nests of germ-cells in early prophase were present at the level of the definitive ovaries of normal females. In 3 of the 7 there were in addition a few oocytes at the commencement of the second growth phase. These rudimentary gonads were more or less deficient in follicle and stroma cells and in the covering of peritoneal epithelium. The nests often appeared syncytial and in an early stage of degeneration. Peritoneal hypertrophy was as widespread as in the cultures treated with the lower concentration of oestradiol, and there was no sign of vasa deferentia.

In the three male-like individuals hypertrophy of the peritoneum was less intense and much more restricted in area, being pronounced only near the level of the posterior limit of the loop of the stomach. There were a few small germ-cells present anteriorly, where the gonads resembled inhibited testes. Vasa deferentia were present posteriorly. In the individual where growth had been least restricted, there were swellings formed from the peritoneal tissue at the level of the posterior limit of the stomach loop, containing many protogones and protogone-like cells in leptotene (Plate 2, fig. M). Comparison with untreated

males indicated that they were genuine enlargements of the testes. The cavities in the swellings were apparently continuous with the vasa deferentia. In the other two males there was complex folding of the peritoneum in this region, but no increase in the number of germ-cells.

Chromophil debris was prominent in the presumed females. In all cases the gall-bladder was collapsed and contained strongly basophil material. It and the hepatic duct were apparently non-functional, as in the other culture treated with oestradiol. The pronephric tubules were much increased in diameter. Those in the hinder part of the mesonephros were considerably larger in diameter and smaller in number than normal.

(d) Culture treated with testosterone

Testosterone had closely similar effects to the lower concentration of oestradiol (Plate 2, fig. N), the only obvious difference being that testosterone did not cause collapse of the gall-bladder. Twelve of the 29 specimens sectioned possessed a few oocytes, some reaching a diameter of $45\ \mu$, and could with some reserve be termed females; 10 more possessing vasa deferentia were assumed to be males. The remaining 7 possessed neither oocytes nor vasa deferentia. Oocytes and protogones often appeared to be deficient in follicle cells, as in the groups treated with oestradiol. In individuals possessing vasa deferentia there was a complex structure formed of folded peritoneum and connective tissue about the level of the posterior limit of the loop of the stomach, but in no case did this contain many germ-cells. In general peritoneal hypertrophy seemed to be less marked in the males than in the remaining individuals. The pronephric tubules were much enlarged in diameter, but the mesonephros was unaffected.

(e) Culture treated with progesterone

(i) *Individuals fixed at age of 200 days.* Of the 14 individuals sectioned, 4 appear to have been female and 8 male, while in the remaining 2 some degree of intersexual development seems to have occurred.

In the *females*, gonadal development had been somewhat inhibited. As in controls of the same age, nests of germinal cells extended further posteriorly than in older females.

In the *males*, on the other hand, although the anterior part of the testes was perhaps retarded in development, the posterior part was enlarged and contained more germinal cells than in the controls. A more notable feature was the presence and large size of vasa deferentia (Plate 2, fig. O). These were much larger than in any of the untreated controls, reaching dimensions in cross-section of $60\ \mu \times 80\ \mu$ in one instance some distance anterior to the pelvic level. They were thus comparable in size to those seen in the much older controls treated with alcohol, and as in the latter culture, the vasa extended forward into the fertile region of the testes.

In the two individuals with intersexual features, nests of their cells with some in leptotene and pachytene, were distributed throughout the fertile part of the gonads, but attained their maximum size posteriorly. Traces of vasa deferentia were present.

(ii) *Individuals fixed at 254 days.* Eleven of the 16 animals sectioned possessed oocytes in the second phase of growth and were probably females. Four of the 5 remaining possessed vasa deferentia and were probably males. In the presumed *females*, the ovaries were greatly reduced in size and were often broken up longitudinally by areas where germinal tissue had disappeared. There was no sign of the formation of ovarian grooves or of an active germinal epithelium. In places where the gonads appeared to be absent, it was sometimes found that deep and slender gonadal folds practically devoid of germ-cells, had fused with the peritoneum of the body-wall. The maximum size of the few oocytes present varied from 25 to 45 μ in individual females, approaching that in the untreated controls of a comparable age (Plate 2, fig. P). A relatively high proportion of the smaller germ-cells showed signs of pycnosis. The gonadal folds were frequently absent behind the level of the pelvic fins, but their position was sometimes marked by slightly hypertrophied peritoneum. A limited amount of hypertrophy and ciliation laterally to the gonadal folds had also occurred anteriorly to the pelvic region in a few individuals.

In the presumed *males* the testes were reduced in size. The vasa deferentia were about as well developed as in untreated controls of comparable age except in one instance where that on the right side was enlarged for a short distance immediately posterior to the hinder limit of the loop of the stomach. In all but two of the individuals sectioned, there was a small or moderate accumulation of deeply chromophil material in the coelomic and other body-fluids. There was no sign of abnormality in either the pro- or the mesonephros in any of the trout treated with progesterone.

(f) *Culture treated with DOCA*

General inhibition of the reproductive organs was even more marked than after treatment with progesterone (Plate 2, fig. Q). Even where present, the gonads were usually broken up longitudinally into discontinuous fragments. Sometimes, isolated oocytes or small pieces of gonadal tissue appeared to be developing in abnormal positions, through fusion of the distal parts of the gonad with the peritoneum followed by the breakdown of the original suspension. When filamentous, the gonads practically lost their identity after fusing with the peritoneum. In one case some of the larger oocytes were found behind the posterior limit of the normal ovary, and oocytes seemed to be unusually widely distributed in a few other individuals.

Oocytes definitely in the second phase of growth were present in 14 of the 20 individuals sectioned. In twelve of these the largest oocytes were of moderate size, varying from 35 to 50 μ in diameter, in the other two they were small. A

small amount of hypertrophied and ciliated peritoneum was seen posteriorly in the gonadal area in a few cases.

Well-developed vasa deferentia were present in three individuals, which were probably genetic males. Neither oocytes nor vasa deferentia were observed in 2 of the 3 instances where the sex could not be determined. In the third case there were vasa deferentia over a short distance in the pelvic region, and an oocyte at the beginning of the second growth phase was seen farther forward. A possible trace of a vas deferens was seen in one individual with fairly large oocytes. If the indeterminate individuals are not considered, the excess of females over males (14:3) is significant. If they are all assumed to be males, the excess is nearly significant.

The coelomic and other body-fluids contained deeply chromophil matter, as in the other experimental groups. There was a considerable quantity of it in ten individuals. There was no sign of any abnormality of the pro- or the mesonephros.

III. *Effects of treatments on the interrenal ('anterior interrenal' of Giacomini)*

In teleosts the probable homologue of the interrenal of tetrapods consists of islands of cells embedded in lymphatic tissue immediately behind the Malpighian capsule of the pronephros (Giacomini, 1910, 1911). Various authors (Padoa, 1942; Witschi, 1953; Segal, 1953; Chang, 1955; Chang & Witschi, 1955) report that oestrogens cause the hypertrophy of the interrenal in tadpoles of *Rana* and *Bufo*.

When histological preparations of the gonads were made the 'anterior interrenal' was included in some individuals. The impression was given that it was hypertrophied, in some cases at least, after treatment with oestradiol and testosterone. But it was not possible to come to a definite conclusion by inspection alone, owing to the diffuseness of the gland, the unavailability of complete serial sections, and the complicating factor that many of the individuals concerned were much retarded in their rate of growth.

An attempt was therefore made to see if the cell volume had increased after treatment. Where it was possible in individuals sectioned at $7\frac{1}{2} \mu$, the number of 'anterior interrenal' nuclei within an area of $13 \mu \times 230 \mu$ ($= 3,000$ sq. μ) was counted. Owing to the small size of islands and their intimate penetration by blood-vessels, the 230μ length usually had to be made up of 3 or 4 shorter lengths from different islands. A summary of the figures obtained is given in Table 5. Owing to the relatively small number of individuals where counts were possible the untreated controls from 206 to 300 days were considered as a single group. The same was done for the animals treated with progesterone aged 200 and 254 days, and for the cultures treated with the higher and lower concentrations of oestradiol. There was no indication in the untreated controls of a change in cell size with age during this period.

The counts for the cultures treated with progesterone and DOCA showed no significant deviation from those for the untreated controls. The mean counts for

the cultures treated with oestradiol was reduced by 29 per cent., that for the culture treated with testosterone by 9 per cent., and that for the alcohol-treated controls by 14 per cent., the second of these reductions being significant and the other two highly significant.

TABLE 5

Number of nuclei per unit volume of the interrenal. The mean number of nuclei per 3,000 sq. μ in sections $7\frac{1}{2}$ μ thick is tabulated. P is the probability that the difference of a mean from the mean of the untreated controls could result from sampling from the same population

Culture	Mean	No of individuals	Standard error	P
Untreated controls	44.7	21	1.37	..
Alcohol-treated controls	38.7	11	1.72	< 0.001
Oestradiol	31.7	12	1.98	< 0.001
Testosterone	40.7	20	2.11	0.05
Progesterone	43.1	19	1.17	0.2
DOCA	44.5	11	2.45	0.9

A reduction in the number of nuclei seen per unit area of section could be caused by a reduction in nuclear size, but the possibility of this factor affecting the readings had been removed by counting only those nuclei where at least half of their volume was within the section. In fact the nuclei seemed to have been little affected, except perhaps in the culture treated with the higher concentration of oestradiol. Here they appeared rather more variable in size and staining reaction than usual, and their maximum diameter was apparently increased from 7 μ to 8 μ , the enlarged nuclei staining rather more deeply than those in the surrounding cells. There was also little change in the amount of intercellular space. In all the cultures the islands of 'anterior interrenal' cells, although irregular in shape, were compact entities surrounded by a connective tissue membrane.

It therefore appears that the 'anterior interrenal' cells were markedly enlarged after treatment with oestradiol, and to a lesser extent in the control culture treated with 0.05 per cent. of alcohol. In the culture treated with testosterone the impression given is that the greater part of the individuals examined were unaffected, but that marked hypertrophy occurred in the remainder. A second count made in the individual of this culture showing the greatest abnormality gave a figure differing little from that of the first count.

DISCUSSION

(a) *The abnormalities in the controls treated with alcohol*

The abnormalities in the young trout treated with alcohol were:

- (1) Hypertrophy of the gonoducts or their equivalent, i.e. precocious development of vasa deferentia in males, and hypertrophy of the peritoneum

lateral to the gonads in females, forming an epithelium homologous in structure and position with that lining the oviducts of other teleosts.

- (2) Some inhibition of oogenesis where this hypertrophy was marked.
- (3) An increased vascularity of the gonads and gonoducts.
- (4) An accumulation of basophil material in the body-fluids.
- (5) Enlargement of the cells of the interrenal.

The first of these abnormalities suggests that there was an unusually large amount of sex hormone in circulation. It is possible that it resulted from contamination by the sex hormones used in the other cultures since Régnier (1942), Witschi (1950), and Bruner & Witschi (1954) have shown that fish and tadpoles are sensitive to extremely small amounts of some sex hormones, concentrations in the order of 0.02 per cent. of the minimum used in the present series of experiments producing marked effects. It is also possible that the alcohol itself was responsible, perhaps through shock action causing the release of sex hormones by the reproductive organs or the interrenal glands, since there have been a number of previous reports of alcohol having a sterol-mimetic action. De Groot and de Wit (1949) reported that, like the sex hormones, alcohol and other narcotic substances, heat shock, and strong light (but not damaging agents in general), cause lengthening of the ovipositor of *Rhodeus amarus*. Stanworth (1953) found that 1% alcohol speeded up the development of nuptial coloration in *Gasterosteus aculeatus* but made no study of the internal effects. He found also that injection of saline or temperature change caused enlargement of the interrenal and changes in the gonads similar in nature to those induced by sex hormones. Jost (personal communication) observed that 2% alcohol in the culture medium had a slight masculinizing effect on tadpoles of *Rana temporaria* and Witschi (personal communication) observed similar effects with 5% of alcohol.

It is to be noted that the distribution of peritoneal hypertrophy in the females of the controls treated with alcohol corresponded with the position of the gonoducts of other teleosts, but that this was not so posteriorly in the cultures treated with oestradiol and testosterone. Furthermore, none of the hormones used, except progesterone in some instances, caused enlargement of the vasa deferentia. Study of adult breeding trout (Ashby, unpublished) has shown that in breeding females, hypertrophy resembling that induced in the experimental cultures is general over the whole of the somatopleure throughout the length of the coelom, except for that part situated medially to the gonads. In ripe males such hypertrophy is almost absent, being limited to a few places immediately lateral to the middle section of the testes. Thus apart from the wider distribution of hypertrophy in adult females, the condition in breeding adults and that in the controls treated with alcohol are comparable.

The present observations and those of Friess (1933) show that in teleosts, adjacent or perhaps overlapping areas of the peritoneum of the gonads hypertrophy to form two very different types of cell, one of these being ciliated and holocrine and secreting into the gonoduct or (in *Salmo*) the coelom, the other

proliferating cells which pass into the ovary. It is possible that the precocious development of ciliated epithelium interfered with the normal formation of the germinal epithelium lining the ovarian grooves, so being a cause of the reduced development of the germinal tissue in the cultures treated with alcohol, oestradiol, and testosterone.

The chromophil deposits in coelomic and other body-fluids found in all experiments were at first thought to be produced by the ciliated holocrine epithelium, since these deposits were greatest in those individuals and groups where hypertrophy of this type was most extensive. But it was found that they were equally large in both sexes, whereas peritoneal hypertrophy was often absent in the males. There was, however, a correlation between the quantity of the deposit and the general sensitivity of the individual to treatment.

(b) The effect of oestradiol and testosterone

Oestradiol and testosterone caused:

- (1) Powerful inhibition of the development of the germinal tissue (with one exception in the group treated with the higher concentration of oestradiol), and an increased vascularity of the gonadal region.
- (2) Widespread hypertrophy of the somatopleure except posteriorly, and except in some males.
- (3) Hypertrophy of the cells of the interrenal more particularly after treatment with oestradiol.
- (4) Enlargement of at least the pronephric tubules of the kidney.
- (5) A great accumulation of basophil material in the body-fluids.
- (6) In the cultures treated with oestradiol, inactivation of the bile-producing system.

The effects were so great that there is no reason to suppose that the alcohol used as solvent could have played an important part in causing them. The close similarity of action of oestradiol and testosterone is remarkable, but is only an addition to the many paradoxical effects of steroid hormones already known. The large amount of peritoneal hypertrophy induced by testosterone, and its virtual absence in adult spawning male trout, might possibly indicate that testosterone is more closely related to the oestrogens of the trout than to androgens.

The inhibitory nature of the effect of these hormones on the germinal tissue could indicate that the hypothesis of Vannini (1950) that oestradiol and testosterone have stimulatory effects on the gonads during differentiation, does not apply generally, but the present work offers insufficient evidence to form a definite opinion. Besides the possible interference with the development of the germinal epithelium already referred to, there is the possibility that the concentrations of hormones used, although suitable for treating anuran tadpoles, were undesirably high for alevins. Padoa (1937) and Berkowitz (1941) found the lower dosages they employed on teleosts to give more inversion and less inhibition. The effects induced by the treatment with oestradiol and with testosterone

in the present work, resemble those observed by Padoa after his more intense treatment of alevins of *Salmo irideus* with oestrone, apart from the fact that he did not refer to hypertrophy of the peritoneum. Experiments with lower concentrations of oestradiol and testosterone have therefore been done, and the effects are now being studied.

The effects of treating juvenile *Salmo* and *Lebistes* with sex hormones resemble those produced in *Ambystoma* to a considerable extent, as Eversole (1939) has already noted in the case of *Lebistes*. Both in fish and urodeles there is much more gonadal inhibition, and much less inversion than in frogs similarly treated. Hypertrophy and ciliation of the peritoneum situated dorso-laterally to the gonads was seen by Mintz (1947) in *Ambystoma* after treatment with testosterone. Similarly Gallien (1954) noted that treatment of *Triton* from an early stage with oestradiol or testosterone, caused the formation of a ciliated epithelium producing mucus, in the area where the internal openings of the oviducts normally form in females. Possibly the hypertrophied peritoneum observed in *Salmo* has evolved from the epithelium of the funnels of the Mullerian ducts, the exact extent of which in teleosts is still in doubt (Goodrich, 1930).

The culture treated with 300 γ /l. of oestradiol did not show any peculiarities, apart from the effect on the mesonephros, which could definitely be attributed to the higher concentration. Although the hypertrophy of the posterior part of the testes in one member was the only instance, apart from the males treated with progesterone and fixed at the age of 200 days, where development of germinal tissue was seen to be stimulated, it is possible that initially stimulation had occurred more widely in the cultures treated with testosterone and oestradiol, but was subsequently obscured by degeneration. Its paradoxical nature recalls the stimulation of spermatogenesis by oestrogen treatment observed in adult teleosts by Castelnovo (1937), Berkowitz (1941), and others.

A failure of bile secretion has not previously been recorded as a result of treatment with oestradiol. It would be possible in some instances to attribute the failure to the poor state of nutrition in the two cultures concerned, but this would not be a satisfactory general explanation as some of the individuals in the other experimental groups had grown less than some of those treated with oestradiol.

(c) *The effect of progesterone and DOCA*

It is evident that progesterone and DOCA can affect gonadal differentiation, as in tetrapods, and that the latter hormone has a more powerful inhibitory action on the reproductive system of trout than of anuran tadpoles. The abnormalities described probably started to develop before the concentration of DOCA was raised to 400 γ /l. since there was little sign of degeneration in progress at the time of fixation.

The excess of females over males in the culture treated with DOCA is the only definite evidence of sexual inversion having occurred in the experiments. The slighter excess of females seen at the end of the experiment with progesterone

seems in view of the greater number of males than females killed at the age of 200 days, to have been due to chance or to a selective mortality of males during the experiments. Even in the culture treated with DOCA, it is possible that the unbalance of the sex ratio was due to selective mortality rather than to sexual inversion, as the survivors numbered less than half of the original culture. Similar considerations apply to the presumed sexual inversion seen after oestrone treatment of alevins by Padoa (1937, 1939a).

Except after treatment with DOCA the incidence of individuals with vasa deferentia in the experimental cultures differed little from that in the untreated controls (where they had not appeared in all males even in the oldest sample examined), being usually present if oocytes were absent, and nearly always completely absent when there were oocytes. If the sexual unbalance in the culture treated with DOCA was due to selective mortality or to chance then there is nothing remarkable in their low incidence in this culture, but if it was due to sexual inversion, there must have been some factor discouraging the development of vasa deferentia where inversion occurred, and permitting their formation where it did not.

The great development of vasa deferentia in the males treated with progesterone which had died at the age of 200 days, was notable in view of their absence in control males of this age. The fact that this hypertrophy was almost totally absent in the males of this culture which survived to the end of the experiments is a further indication that a selective mortality may have occurred. But when full allowance has been made for the exceptional cases, the impression given in all cultures is that the vasa deferentia were remarkably little affected by the hormones administered or by the inhibition of the gonadal development.

(d) *The effect of hormones on the interrenal ('anterior interrenal') and the kidneys*

Cell counts on 'anterior interrenal' were made difficult by its diffuseness and small size and the fact that care was needed to distinguish 'anterior interrenal' from pronephric cells. In addition the nuclei of the islands of 'anterior interrenal' cells were usually more concentrated peripherally than centrally, and in some individuals the nuclei were more concentrated in some islands than in others. The effect of the latter factors was minimized by choosing islands for counting at random and using as far as possible a standard counting procedure. The fact that the mean in two of the experimental cultures differed little from that in the untreated controls increases the probability that the method used was reliable.

Stanworth (1953), as in the present experiment, has observed an oestrogen (oestrone injected in saline) to have a powerful hypertrophic effect on the 'anterior interrenal' of a teleost *Gasterosteus aculeatus* L. Testosterone (injected in oil) had no such effect, but some enlargement occurred with progesterone, these two results being different from those observed in *Salmo*. The hypertrophic effect perhaps produced by alcohol in the present experiments and that produced

by saline injections and temperature change in *Gasterosteus* have already been compared. Stanworth did not see any reaction following these treatments in the Corpuscles of Stannius (sometimes called the 'posterior interrenal'), which develop as buds from the mesonephric ducts. The similarity of the response of the 'anterior interrenal' of teleosts and of the interrenal of tetrapods to steroid hormones and other treatments, tends to confirm as Stanworth has said, that Giacomini was correct in suggesting the 'anterior interrenal' rather than the Corpuscles of Stannius to be the equivalent of the interrenal of other vertebrates, and until there is firm evidence available that the Corpuscles of Stannius in fact have interrenal affinities, the 'anterior interrenal' might well be termed simply the 'interrenal'. This has a certain bearing on the controversy over the origin of the interrenal. Vannini and his supporters claim that in tetrapods (see Vannini & Sabbadin, 1954) and in elasmobranchs (Chieffi, 1952), the interrenal arises, like the 'anterior interrenal' of teleosts, from the ventral mesoderm at the apex of the mesentery, whereas the school of Witschi (see Witschi, 1953; Segal, 1953) believes it to be derived, like the kidney, from the intermediate mesoderm.

Witschi reports that the enlargement of the interrenal seen in tadpoles after treatment with oestradiol is due largely to the conversion of cells of the mesonephric blastema into interrenal cells. Owing to the mesonephros being much posterior to the 'anterior interrenal' in teleosts, this could not have occurred in the present experiments, and there was no evidence of the transformation into 'anterior interrenal' tissue of cells of the pronephros, the tubules of which were already differentiated at the beginning of the experiments. Neither could there have been any conversion of gonadal into mesonephric tissue as is reported to occur by Bruner & Witschi (1954) in *Ambystoma* larvae when treated with testosterone, since the gonads and kidney were widely separated by the swim-bladder throughout the experiments. The effects on the gonads, interrenal and kidney, produced by the sex hormones in *Salmo* were induced independently of any close spatial relationship between these three organs such as is seen in amphibia.

(e) *Mode of action of the hormones*

The fact that the interrenal was affected increases *a priori* the likelihood of the pituitary having been involved in the observed modifications in gonadal development. Bretschneider & de Wit (1947) believe that mammalian sex hormones affect the reproductive system of teleosts only in an indirect manner via the hypophysis. But it has been generally concluded in tetrapods that the reproductive system develops independently of the pituitary until gonadal differentiation has occurred. Witschi (1953) and Chang & Witschi (1955) find that the effects of oestradiol on the gonads of tadpoles of *Rana* is not affected by hypophysectomy, but that hypertrophy of the interrenal is prevented.

The results of the experiments do not throw much light on the problem of

whether sex hormones induce sex reversal in juvenile vertebrates by acting as sexual inductors or by interfering with the action of those normally produced in embryos and larvae. But the slight transitory hypertrophy and ciliation of the peritoneum seen in female untreated controls during the later stages of gonadal differentiation perhaps indicates that sex hormones were being formed at this stage.

SUMMARY

1. Steroid hormones were administered to trout alevins by adding them to the culture medium in alcoholic solution, the concentration of hormone in the cultures being within the range 50 γ to 400 γ /l., and that of alcohol, 0.05% to 0.3%. A control culture was maintained in 0.5% of alcohol.

2. Oestradiol (50 γ and 300 γ /l.) and testosterone (60 γ /l.) had closely similar effects, which included strong inhibition of the development of germinal tissue except in one male treated with the higher oestradiol concentration, where stimulation occurred posteriorly. There was no evidence of sexual inversion, and vasa deferentia appeared in a normal proportion of individuals. With the partial exception of some males, a large area of the somatopleure in the individuals treated became, as in female breeding trout, a ciliated columnar epithelium resembling the internal epithelium of the gonoducts of other teleosts. The connective tissue of the gonadal area was unusually vascular, and in the body-fluids there were large amounts of deeply chromophil material. The tubules of the pronephros were much enlarged. Those of the mesonephros were increased in diameter and reduced in number in the culture treated with the higher concentration of oestradiol. Both concentrations of oestradiol caused the collapse of the gall-bladder, this being the only obvious difference from the effect of testosterone. There are strong reasons for believing that oestradiol caused enlargement of the cells of the interrenal ('anterior interrenal') gland, and some reason to think that in some instances testosterone could also cause their hypertrophy.

3. Progesterone and more particularly desoxycorticosterone acetate inhibited the development of germinal tissue, except in some males treated with the former hormone, where in addition vasa deferentia developed precociously and were much enlarged. Vasa deferentia of a normal size were present in most other individuals lacking oocytes. There was a significant excess of females over males after treatment with DOCA, but the possibility of this being due to a selective mortality cannot be ruled out. There was evidence of such a mortality having occurred in the culture treated with progesterone. Deeply chromophil material was usually present in the body-fluids.

4. Slight sterol-mimetic effects were observed in the controls treated with alcohol. Possible causes are discussed.

5. The results are compared with those obtained from similar experiments in other teleosts and in tetrapods.

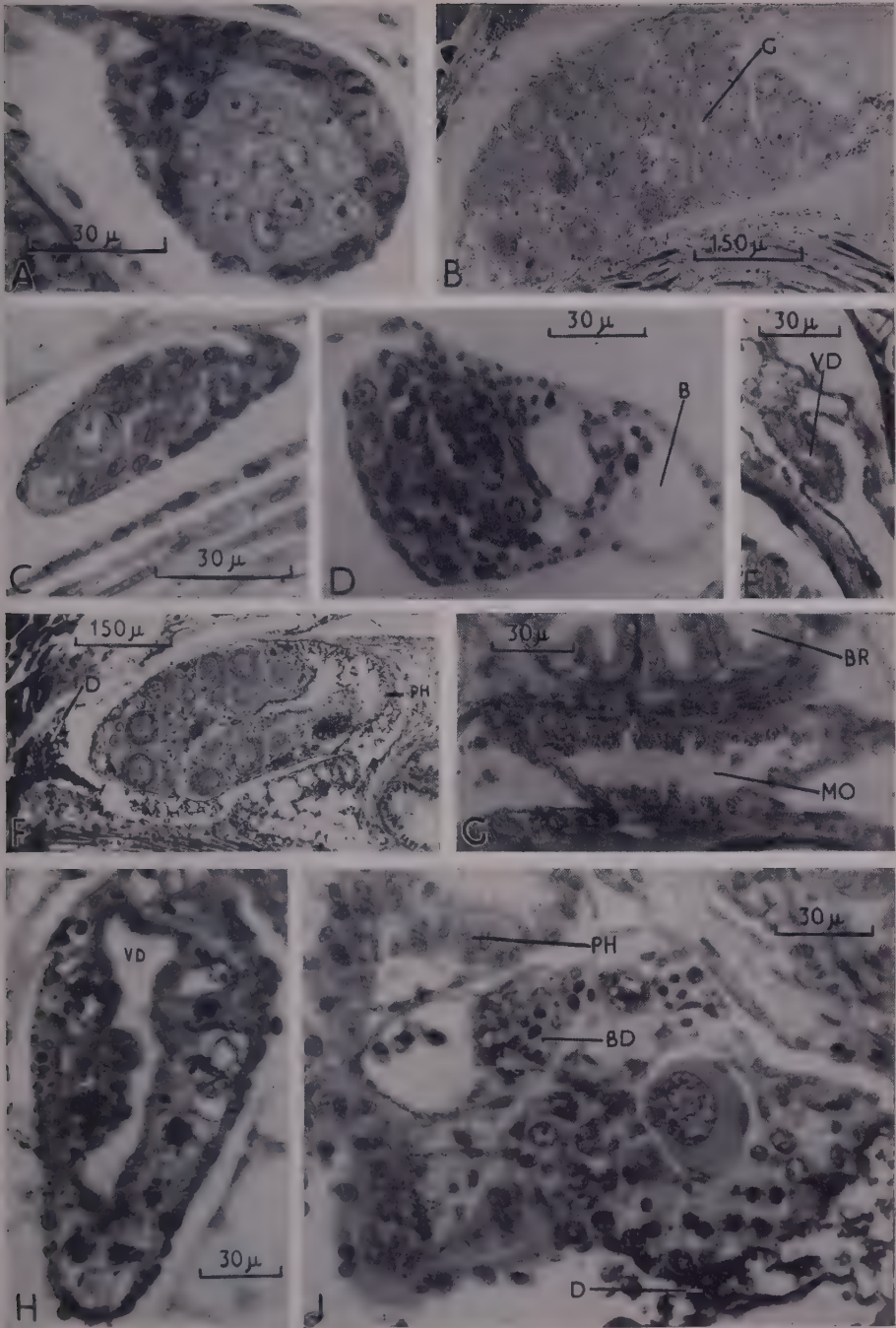
ACKNOWLEDGEMENTS

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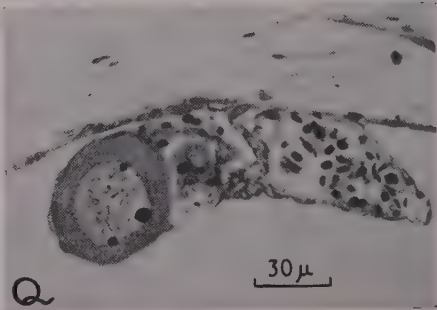
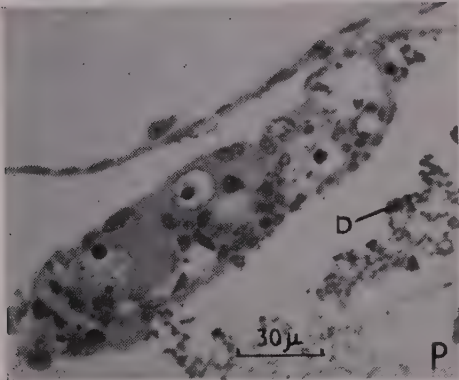
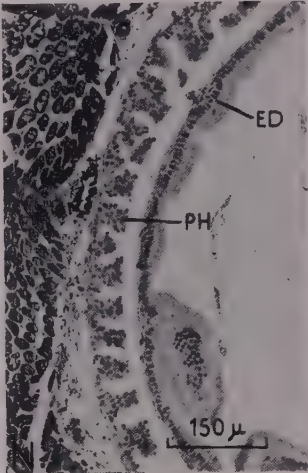
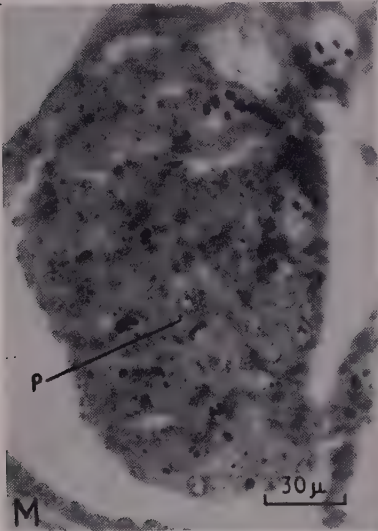
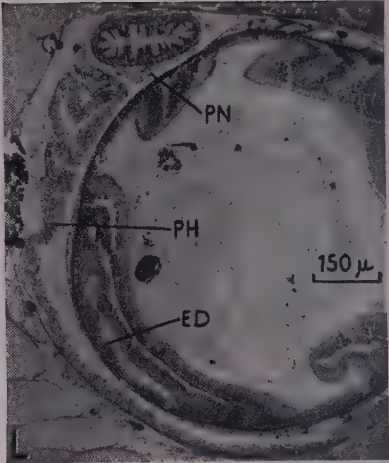
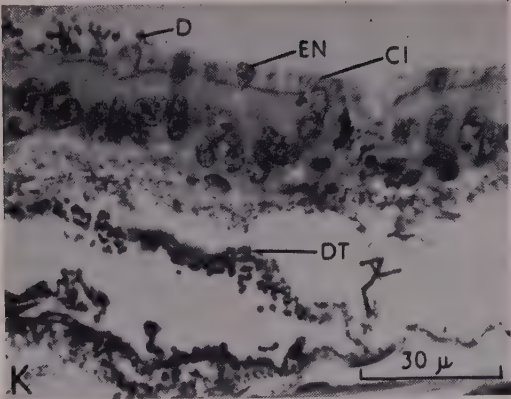
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K. R. ASHBY

Plate 1



K. R. ASHBY

Plate 2

EXPLANATION OF PLATES

All figures are of transverse sections with animal's right on right-hand side.

Abbreviations: B, blood-vessel; BD, enlarged blood-vessel with chromophil deposit in plasma; BR, bladder; CI, cilia; D, chromophil deposit in coelom; DT, chromophil deposit in tissue fluid; ED, endoderm; EN, extruded nucleus; G, groove on germinal side of ovary; MO, median oviduct; P, protogone; PH, hypertrophied peritoneum; PN, peritoneum never hypertrophied; VD, vas deferens.

PLATE 1

Untreated controls

FIG. A. Ovary at 169 days containing large nest of protogones.

FIG. B. Ovary at 300 days.

FIG. C. Testis at 169 days.

FIG. D. Testis at 300 days.

FIG. E. Vas deferens at pelvic level at 300 days.

Controls treated with alcohol

FIG. F. Ovary.

FIG. G. Terminal median oviduct.

FIG. H. Vas deferens anterior to pelvic level.

Oestradiol 50 γ /l.

FIG. J. Reduced ovary.

PLATE 2

Oestradiol 50 γ /l.

FIG. K. Hypertrophied peritoneum.

FIG. L. Body-cavity somewhat anterior to pelvic level showing general hypertrophy of peritoneum.

Oestradiol 300 γ /l.

FIG. M. Posterior enlargement of testis.

Testosterone

FIG. N. Somatopleure at pelvic level showing general hypertrophy and folding.

Progesterone

FIG. O. Enlarged vas deferens at pelvic level in male aged 200 days.

FIG. P. Reduced ovary of female aged 254 days.

DOCA

FIG. Q. Reduced ovary.

(*Manuscript received 26 : vii : 56*)

The Pulmonary Return as an Agent in the Final Development of the Atrium in *Rana Temporaria*

by J. R. SIMONS¹

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INTRODUCTION

NOBLE (1925) concluded that the fenestration of the interauricular septum and the small size of the left auricle in many urodeles were to be correlated with the reduction of the importance of pulmonary respiration in the group. He argued that the reduction in lung breathing conditioned a reduction in the pulmonary return to the heart, in which the left auricle became reduced in size and the interauricular septum fenestrated. He suggested that experiments might be designed to test this theory, but a search of the literature reveals that no such work has ever been reported.

In the early stages of the frog tadpole the heart is provided with a single-chambered atrium which later becomes divided into right and left auricles by the growth of the interauricular septum. Before the development of this septum, however, the lungs become established and the possibility therefore arises of interfering with and obliterating the pulmonary return before the atrium becomes divided. Such a procedure would, it was felt, be the type of experiment that Noble had in mind to test his thesis, and therefore the work described herein was initiated in order to determine what effect lung extirpation had on the development of the septum and the auricles in the tadpole of the frog, *Rana temporaria*.

MATERIAL AND METHODS

The tadpoles used in this present study were hatched from spawn obtained from laboratory specimens of *R. temporaria*. They had just entered the fifth (12 mm.) stage of Marshall & Bles (1890), that is, immediately after the atrophy of the external gills. At this stage the lungs are present, as also are the pulmonary veins, but the interauricular septum has not yet begun its development. This begins some 4 or 5 days after the disappearance of the external gills. It appears as a fringe of tissue growing down from the dorsal wall of the atrium to meet the partition developing between the atrium and the ventricle.

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Tadpoles were anaesthetized by being placed in a 2 per cent. solution of urethane. They were then placed under a binocular microscope and, by means of a fine scalpel, two slits were cut in the body-wall, one on each side of and parallel to the mid-dorsal line. The lungs are a pair of small silvery sacs and readily come to the surface through such slits. Anteriorly the lungs are joined to the oesophagus by a short laryngo-tracheal tube which is identifiable as being heavily pigmented.

When the lung extruded sufficiently through the aperture it was grasped with fine forceps and pulled until the pigmented laryngo-tracheal tube came into view. This was cut across by fine scissors. Both lungs were treated similarly. The operation concluded, the animal was dropped into a beaker of water. It was kept separated from its companions overnight and, if still alive in the morning, transferred to an aquarium with other operated animals. A second aquarium contained tadpoles to be used as unoperated controls. Both control and experimental animals were fed on raw and cooked meats.

At intervals between the operation and metamorphosis, tadpoles were randomly selected and fixed in Bouin's fixative. Three groups were formed by the selection: group 1 containing tadpoles having incipient limb-buds, group 2 containing tadpoles with well-developed hind-limbs, and group 3 containing animals either at the point of metamorphosis or within 2 days after. The fixed material was prepared as serial sections of thickness $8\ \mu$ and stained with Ehrlich's haematoxylin and eosin. The sections were projected at a magnification of 62.5 diameters, and the outlines of the projected images of each fifth section of the auricles drawn by following the inner line of the wall of the auricle with a fine-pointed pencil. The areas of the outlines so obtained were computed with the aid of a planimeter.

RESULTS

(a) *General*

The mortality rate was very high. Of a total of 135 animals operated upon only 32 survived to be fixed and sectioned.

The rate of development of the experimental animals was much slower than of the control group. Thus, of a group hatched at the beginning of February, the controls had all metamorphosed by the end of March. The first experimental animals to metamorphose did so in the second week of April. In the first week of June some experimental tadpoles had only reached the stage of having well-developed hind-limbs.

As well as taking longer to reach any given stage, the experimental animals were generally smaller than the controls at the same stage. This was very noticeable in the first few weeks after the operation had been performed. Thus, the experimental animals of group 1 averaged only two-thirds of the length of the controls of the group. As development proceeded this size difference disappeared.

Because the lungs did not regenerate, the experimental animals when gulping

air tended to pass it into the general body-cavity from whence they were unable to expel it. This caused them to have an unnatural buoyancy and they consequently spent much time floating helplessly at the surface. Generally, within 4 or 5 hours the trapped air was dispelled.

It is interesting to note that, after metamorphosis, the experimental animals appeared to 'gasp' at intervals and to hold their mouths open for a considerable time. Such behaviour was not seen in the recently metamorphosed control tadpoles.

(b) *Measurements*

The absolute and relative volumes of the auricles of twenty-five experimental and fifteen control animals were measured.

In the first group (animals with incipient limb-buds) the average volume of the total atrial lumen of eight experimental animals was 0.11 mm.^3 and the volume of the lumen of the left auricle averaged 19.7 per cent. of the volume of the total atrium. The respective values in four control animals were 0.93 mm.^3 and 25.0 per cent. In the second group (eleven experimentals and six controls) the values for the total atrial volume and the left auricle as a percentage of this were 0.28 mm.^3 and 16.0 per cent. respectively in operated animals, and 0.58 mm.^3 and 31.9 per cent. respectively in controls. In the third group (six experimentals and five controls) the values were 0.31 mm.^3 and 20.3 per cent. respectively in operated animals, and 0.52 mm.^3 and 33.8 per cent. respectively in controls.

The relative volume of the lumen of the left auricle is thus in each group much smaller in the experimental animals than in the controls and, over the whole assemblage of groups, averaged 18.2 per cent. as against 30.7 per cent. Statistically this result is highly significant ($t = 5.97$, d.f. = 38, $P < 0.01$).

The relative volume of the lumen of the left auricle in the control animals increased from 25.0 per cent. (group 1) to 33.8 per cent. at metamorphosis. This increase is just significant ($t = 2.33$, d.f. = 7, P just greater than 0.05). On the other hand, in the experimental animals the difference between the relative volumes of the lumen of the left auricle in the animals of group 1 and those at metamorphosis is not significantly different. It is thus concluded that the lumen of the left auricle in the experimental animals, although increasing in absolute size as the animals developed, failed to increase its volume relative to the lumen of the atrium as a whole but remained relatively and significantly smaller than did the lumen of the left auricle of the control tadpoles.

The absolute volume of the lumen of the whole atrium of the experimental animals was, in each group, smaller than that of the controls. This difference was most marked in the youngest animals (group 1) and was undoubtedly due to the general retardation of growth seen in the younger animals, quite apart from any effects of the destruction of the pulmonary return. In the later experimental animals, the absolute volume of the lumen of the whole atrium came closer to

that of the control animals in the respective groups and was not significantly different from it.

(c) *Microscopical examination*

The microscopical appearance of the heart sections showed that occasionally a small pulmonary vein was present in the experimental animals, but that it disappeared after running a short distance into the remnant of the laryngo-tracheal tube. In the majority of cases, however, there was no doubt that the pulmonary return to the heart had been obliterated. In all other respects (i.e. the thickness of the auricular walls, the developing interauricular septum, and the general configuration) the experimental hearts were similar to the hearts of the control animals.

The first sign of the interauricular septum was found one week after the removal of the lungs as a tongue-like rudiment hanging from the dorsal wall of the atrium and pointing towards the valveless atrio-ventricular orifice. The appearance and configuration of the fully developed interauricular septa of the experimental animals were completely normal, as was also the case with the control animals. There were, however, three instances of auricles and septa of abnormal appearance in experimental animals (not included in the assembly from which the data so far presented were measured). It is considered that these abnormalities were due to the mechanical damage done to the sinu-atrial region of the heart when the lungs were removed.

DISCUSSION

The results show that, when the pulmonary return is obliterated, both the absolute and proportional volumes of the left auricular lumen in operated specimens are smaller than in the control animals.

Stohr (1925, 1927) found that, when the blood circulation of the embryos of *Triton taeniatus* and *Bombinator pachypus* was obliterated, the heart anlage developed small but normally shaped hearts. On the other hand, Fales (1946), working on embryos of *Ambystoma punctatum*, found that depriving them of their circulation caused the hearts to develop chambers with abnormally thick walls. In the present experiments there was no sign of any unusual thickening of the walls of any of the heart chambers.

The influence of the flow of the blood through the atrium on the development of the interauricular septum has long been a matter of interest. Bremer (1928) considered that, since the septum in the hearts of chicks and turtles developed transversely across the line of flow, it was not influenced by the blood-stream. Goerttler (1955) concluded that the pulmonary return played no part in the genesis of the septum but might possibly influence its later development. This latter view is confirmed in the present work in so far as the interauricular septum's origin is concerned, but the later stages of development appear also to be equally independent of the pulmonary return.

Noble (1925) advanced the thesis that the fenestrated interauricular septum found in many urodeles is due to the tendency in these animals to elaborate methods of respiration other than pulmonary. The process is to be conceived as an evolutionary adaptation to an amphibian life in an 'essentially moist and cold environment', in which first the left auricle became reduced in size, then the interauricular septum broke down, and finally the spiral valve disappeared. No frog or toad is known to lack a spiral valve or to have a fenestrated interauricular septum. On the other hand, two frogs occur (*Astylosternus robustus* and *Batrachophrynus brachydactylus* described by Noble, 1925) that have comparatively small left auricles, small lungs and the cutaneous respiratory surfaces greatly increased by various means.

Noble suggested that these two frogs were in the first phase of an evolution which would eventually bring about the loss of the interatrial septum, and his supposition that the left auricle had been reduced in conformity with the small lungs would agree with the experimental results detailed in this work on *Rana temporaria*.

SUMMARY

1. The lungs of tadpoles of *R. temporaria* were removed at an early stage (12 mm.) in order to ascertain the effect on the development of the atrium and the interauricular septum of the heart. Serial sections of the hearts of experimental and control tadpoles at three comparable stages of development between the operation and metamorphosis were prepared.

2. The operation causes a reduction in the absolute volume of the atrium.

3. When it is first formed, the left auricle in the normal tadpole averages 25 per cent. of the total atrial volume but, as growth proceeds, it comes to average about one-third of the total atrial volume by the time metamorphosis occurs. In the experimental tadpoles the left auricle, when first formed, averaged about one-fifth of the total atrial volume and maintained a similar proportion throughout development to metamorphosis. The presence of the pulmonary return is thus necessary for the normal enlargement of the proportional volume of the left auricle.

4. The obliteration of the pulmonary return did not affect the origin and normal development of the interauricular septum.

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Myotypic Respecification of Regenerated Nerve-fibres in Cichlid Fishes¹

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INTRODUCTION

IN man and other mammals normal motor co-ordination is not restored, as a rule, after regeneration of a severed peripheral nerve-trunk (Sperry, 1945). The random misdirection of regenerating fibres into foreign muscles tends to prevent normal dissociated action within the re-innervated musculature. In contrast, larval amphibians have been found to show excellent recovery of motor function in the form of 'homologous or myotypic response' (Weiss, 1936, 1941) following the cutting and regeneration of limb-nerves, limb transplantation, and the cross-connecting of limb nerves to foreign muscles. Similarly, good restoration of muscle co-ordination has been observed in the pectoral fin of adult teleost fishes (Sperry, 1950, 1956).

It has been suggested (Sperry, 1941, 1951) that such recovery is most easily explained in terms of a central readjustment of synaptic connexions to suit the altered pattern of peripheral innervation. Morphological or other direct evidence for such synaptic changes, however, has not been found. For further investigation of the problem fishes appear to offer promising possibilities in that the technical difficulties of staining the synaptic terminals in fishes appear to be somewhat less formidable than in the amphibians.

The following study was undertaken to ascertain whether the type of motor recovery observed in the pectoral fin centres of adult teleosts might also occur in a cranial nucleus where the conditions for histological demonstration of synaptic end-feet are much more favourable. The experiment involves regeneration following surgical section and cross-union of branches of the mandibular ramus of cranial nerve V in the cichlid fish, *Astronotus ocellatus* (Agassiz), with a study of the extent of subsequent functional recovery. The results demonstrate motor readjustment in the trigeminal system similar to that reported previously for the innervation of limb and fin musculature in amphibians and fishes respectively.

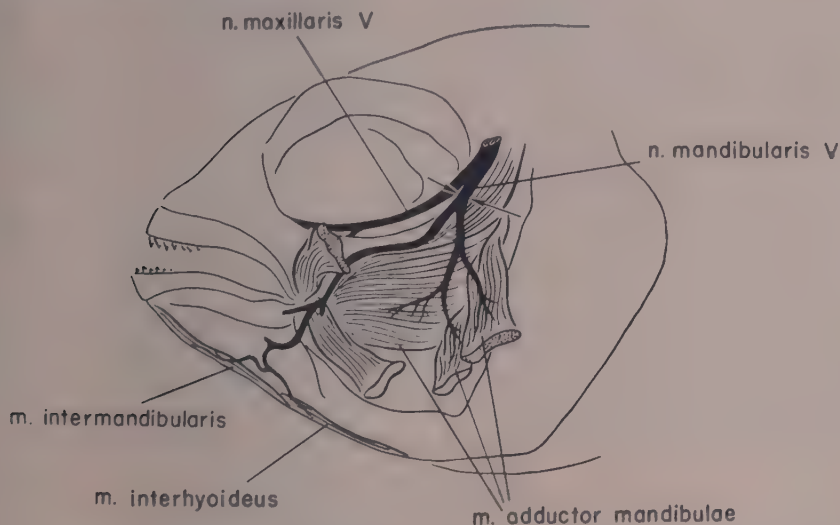
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MATERIALS AND METHODS

Mandibular muscles and their innervation

The closing and opening of the mouth in *Astronotus* is governed by two sets of antagonistic muscles which act to elevate and depress the mandible respectively. The closing of the jaw is effected by a set of muscles, the adductor, or levator, mandibulae, which in this species has three subdivisions (Text-fig. 1). The opening of the jaw is controlled by the intermandibularis and the interhyoideus muscles. The posterior portion of the intermandibularis is fused with the interhyoideus, thus forming a common sheet of muscle that pulls the mandible downward. The latter muscles will be referred to here as the 'depressors' and the former as the 'levators'.



TEXT-FIG. 1. Sketch of jaw musculature and its innervation in *Astronotus ocellatus*. The two superficial adductor muscles are cut and deflected to expose the deeper adductor muscle. Arrows indicate point of section of mandibularis V nerve in Series I.

Both the levator and depressor muscles are innervated by the *ramus mandibularis trigemini*. The ramus mandibularis receives centrally all of the motor-fibres of V and some sensory fibres and, soon after its emergence from the cranium, gives off a motor-bundle (Text-fig. 1) to the levator muscles of the mandible. The nerve then turns laterally and ventrally into the lower jaw and divides into several branchlets, one of which carries all of the remaining motor-fibres and supplies the depressor muscles.

Surgical procedure

The fish were anesthetized by placing them in a solution of Tricane (MS 222, Sandoz Chemical Co.) mixed in the proportion of 0.05 grammes per litre of

conditioned tap-water. The surgery was performed with the fish out of water under the dissection microscope with a magnification of 10 times. The anesthetized fish, wrapped in a wet cloth, was placed in a plasticene mould padded with gauze to hold it in the desired position. Anesthesia was maintained by dropping slowly over the gills a diluted solution of Tricane.

The nerve-sections were performed within the orbit. The conjunctiva was cut on the ventral side to free the eyeball, which then was raised and retracted gently to the dorsal edge of the orbit. This procedure exposed the infraorbital nerve-trunk which runs laterally along the floor of the orbit. The infraorbital trunk in this region consists of three main divisions, the middle one being the mandibularis illustrated in Text-fig. 1. Section of the nerves was done with fine scissors and forceps. The nerve-ends were reunited by simply apposing them and allowing a few moments for coagulation between the severed ends. Later the eyeball was replaced and the fish were put in a beaker of aerated aquarium water until they had recovered, after which they were returned to aquaria, two fishes per 3-gallon tank. The tanks were continuously aerated and the water temperature was thermostatically controlled at approximately 25° C. Penicillin was added to the tanks, 40,000 units per gallon, along with quinine hydrochloride, 1 grain per gallon, on the day of operation to minimize postoperative infection.

After regeneration and subsequent testing of functional recovery, the operated region was examined again under anesthesia, the surgical procedure being similar to that described above.

The fishes used in this experiment measured 4–6 cm. in total length at the time of operation, and they increased approximately 0.5 cm. in length during the period of observation, roughly 4 weeks. These specimens were quite young; the adults reach a length of 20–25 cm.

OBSERVATIONS

Series I. Proximal section and regeneration of mandibular trunk

In ten fish, the left mandibular nerve was completely divided at a point proximal to its bifurcation into levator and depressor branches (level of section is marked by arrows in Text-fig. 1). Before their reapposition the two ends were teased open to favour a random disarrangement of the interneuronal fibre-pattern in regeneration.

Partial paralysis was visible on the left side during the first week following the operation. However, since the two mandibles are fused in front, the whole lower jaw continued to move up and down in a near-normal fashion owing to the action of the muscles on the right side. The fish was able to ingest and masticate its food, including whole snails and small bits of meat, much as usual.

In one specimen of this series the mandibles were separated at the time of operation by an incision at the symphysis extending ventrally and medially through the intermandibularis muscle-plate. In this case the left mandible hung

open and immobile except for a slow feeble tremor associated with the action of the opercular and branchiostegal muscles mediated by nerve VII. This residual movement, which was not sufficient to enable the fish to close the mandible, disappeared when the hyoid and the mandible were disconnected from the operculum and the branchiostegal rays.

The right mandible, meanwhile, continued to move up and down normally, and the fish was able to pick up and eat very small pieces of meat. This separation of the mandibles was not carried out in the other specimens at this time, because it had been found that it results, in the course of growth, in an abnormal curvature of the lower jaws that renders post-recovery observations difficult.

The first signs of recovery in the specimen with divided mandibles appeared on about the 7th day after operation. By the 10th day there were definite movements on the operated side, and by the 16th day the mandibular movement had been almost completely recovered. The recovered movement when first detected on the paralysed side was timed synchronously with that of the normal right side. The abnormal growth of the mandibles during the subsequent recovery period made further observations difficult. In the other fish of the series also the paralysis on the left side was no longer visible by the 16th day.

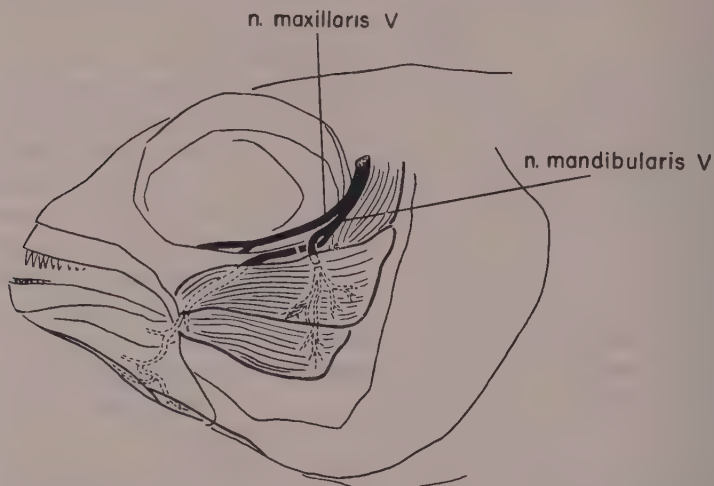
On the 21st day after operation the right mandibular nerve was cut in four specimens of the series in a position corresponding to that of the original section on the left side. In all four cases the movement of the lower jaw, activated now through the regenerated left nerve, was near normal except for a slight paralysis on the right side produced by the nerve-section on that side. The fish were able to pick up and chew food without difficulty. Two days later the regenerated left mandibular nerve in these four cases was cut at the site of the scar. This left the lower jaw hanging open and almost immobile except for the residual movement referred to above. The fish were unable to close the mouth or to feed. The results were the same in all four specimens.

In the remaining five specimens the two mandibles were separated by a medial incision at the symphysis on the 24th day after operation, in order that the independent movement of the two halves of the jaw might be compared for timing and co-ordination. In all cases the two mandibles were found to open and close with perfect synchrony, indicating normal timing in the re-innervated muscles. On the following day when the right mandibular nerve was cut, the right mandible hung open but the left continued to move normally. Two days later the regenerated left mandibular nerve was cut at the site of the scar in two of the latter five specimens, resulting in paralysis of the lower jaw except for the slight residual movement noted previously.

Series II. Cross-union of levator and depressor nerves

In the second series of operations the left mandibular nerve was sectioned distal to its bifurcation; that is, the levator and depressor branches were cut separately. The four nerve-stumps were then cross-united so that the levator

fibres would grow back into the depressor muscles and the depressor fibres into the levator muscles as indicated in Text-fig. 2. Fourteen fishes were operated, eleven of which survived for the testing of functional recovery.



TEXT-FIG. 2. Sketch showing cross-union of levator and depressor branches of mandibularis V nerve.

The visible effects of the operation were the same as in Series I. In two of the specimens the mandibles were separated at the time of the operation to facilitate detection of the first signs of recovery. On about the 8th day after operation, slight movement was observed in the paralysed left mandible of these two cases. By the 14th day recovery was pronounced, though the movement of the two separated mandibles was restricted because the mandibles had grown to overlap anteriorly.

Checks for recovery were carried out as in Series I, beginning on the 23rd to 29th days after operation. In four cases the right (unoperated) mandibular nerve was cut. Three of the four showed partial paralysis of the right mandible as noted in Series I, but without serious impairment of the movement of the jaw which now was activated by the regenerated crossed nerves. The fourth specimen had already shown a lack of complete recovery in that the slight paralysis observed after the original operation had not been corrected. The downward movement in the left mandible was extremely weak and this fish was hardly able to ingest usual-sized pieces of food, snails, or bits of meat. Unlike the three normally recovered specimens, it had to make repeated attempts to pick a piece of food from the bottom of the tank. After the right mandibular nerve was sectioned in this specimen, the jaw remained in a nearly locked position. An examination of the regenerated left mandibular nerve revealed that the levator branch had grown back towards its own stump, along with the crossed depressor, apparently

leaving the distal stump of the depressor branch without any motor connexion. The operated region of the three normally recovered specimens was then examined and in all these cases the cross-union of levator and depressor branches was complete and the nerves had grown into the foreign muscles.

In five other fish of Series II the two mandibles were separated at the symphysis so that the function of each could be observed independently. In four of these specimens the right and left mandibles moved up and down with perfect co-ordination and timing. The movement on the left (regenerated) side was normal except that the mandible did not close quite as tightly as did the right mandible. Sectioning of the right (unoperated) mandibular nerve in these four specimens produced paralysis of the right mandible, but the fish continued to use the left mandible in approximately normal manner. On separation of the mandibles in the fifth specimen, it was found that the movement of the left mandible was impaired. When the right mandibular nerve was cut, the right mandible hung open and the left mandible was locked in an almost closed position. Examination of the regenerated nerve revealed that in this exception the levator branch had grown back towards its own stump, instead of into the depressor stump as intended.

The regenerated nerve in the four completely recovered specimens was then examined by dissection under anesthesia. In one case the levator and depressor branches were found to have slipped and grown back into their own respective muscles. In the other three specimens the cross-union of levator and depressor branches was complete. Careful examination was made to ensure that no stray fibres connected the levator and depressor branches to their own respective muscles. A further electrical check was performed in which the connexion between the levator branch and the depressor stump was severed, leaving the other cross-connexion (depressor branch into levator stump) intact. The mandibular nerve was then lifted from the surrounding tissue by means of a curved needle, and electric stimulation from an induction coil was applied at a point above the scar. The result was a pronounced contraction in the levator muscles, indicating that functional connexion had been established between the depressor nerve components and the levator muscles. Sectioning the depressor branch at the site of the scar eliminated all response in the levator muscles.

The two specimens in which the mandibles had been separated at the time of operation were not tested further because of the lopsided regrowth of the mandibles.

DISCUSSION

The recovery of normal vigorous movement of the mandible observed in Series I could have been effected only through re-innervation of the paralysed mandibular muscles and activation of these muscles in their proper timing. This means either (*a*) that the regenerating motor-fibres must have re-established their functional connexions selectively with their original muscles, or (*b*) that those

fibres that connected with foreign muscles must have changed their central timing to suit the new terminations. The latter alternative is strongly favoured by the occurrence of similar recovery in the second series in which the nerve-fibres were definitely cross-connected by surgical means to antagonist muscles. In view of the rapidity of recovery in Series II and the results of the combined physiological and anatomical checks, it is extremely improbable that the mandibular muscles could have become re-innervated by their original motoneurons.

The possibility that the central readjustment in timing could have been effected on a reflex or other purely functional basis such as learning is contra-indicated by the fact that such readjustment has not been observed to occur in other vertebrates including man. Nor was there any visible evidence of re-learning during the recovery of mandibular movement. Accordingly it is inferred that the trigeminal motoneurons automatically adjusted their central timing to suit whatever trigeminal muscles their regenerating axons happened to connect with in the periphery. This is the type of motor re-adaptation observed originally by Weiss (1936, 1941) in the limb innervation in larval urodeles and later in newly metamorphosed toads, and found more recently in the limb innervation of adult *Triturus* (Sperry, unpublished) and in the fin innervation of adult fishes (Sperry, 1950, 1956). Investigation has failed to reveal this kind of readjustment in the limb innervation of adult frogs, in the oculomotor system of the adult swell-fish, and of mid-larval and adult amphibians, and in the motor system generally of young and adult mammals (Sperry, 1945, 1947, 1956, and unpublished data).

Following the early proposal of Wiersma (1931), it has been accepted that the exceptionally good motor re-adaptation where it has been found in fish and amphibians must involve a transmission of some kind of specifying influence from the muscle to the regenerated motor axons. Exactly how the specification imposed upon the nerve axon by the muscle subsequently affects the incidence of central discharge remains to be determined. The possibility of checking experimentally the suggestion that the central timing is changed through a readjustment in the synaptic end-feet on the affected motor neurons is rendered somewhat more feasible by the present finding that such motor readjustment can occur in a cranial motor-nerve nucleus in postembryonic stages.

SUMMARY

1. The mandibular portion of cranial nerve V of the teleost fish, *Astronotus ocellatus*, was totally severed and allowed to regenerate into its distal nerve-stump in a series of ten cases.

2. In a second series of fourteen cases, the two nerve branches supplying the elevator and depressor muscles of the mandible were sectioned separately and the nerve of the depressor muscles was crossed into the levator muscles and vice versa.

3. In both series, return of function in the re-innervated muscles was first

detected about 8 days after nerve-section, and full recovery was achieved by the 18th to 21st day save for two exceptions in Series II in which the surgical cross-union proved faulty.

4. The recovered mandibular action was properly timed and co-ordinated from the beginning with the mandibular muscles of the opposite side.

5. It is inferred (a) that the regenerating motor-fibres establish functional connexions with foreign mandibular muscles, (b) that the muscles induce a local muscle specificity in the regenerated motor axons, and (c) that this 'myotypic' specificity determines in some way not yet analysed the central timing of moto-neuron discharge in the trigeminal motor nucleus.

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Différenciation *in vitro* de blastodermes de Truite (*Salmo irideus*) dépourvus de couche enveloppante

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L'ÉTUDE structurale et fonctionnelle du blastoderme de Poisson conduit à y distinguer deux ensembles: la couche enveloppante et les blastomères profonds. Si du seul point de vue quantitatif (volume total des cellules) la couche enveloppante est beaucoup moins importante que la masse blastomérienne, du point de vue qualitatif elle représente un élément essentiel dans le développement du blastoderme depuis le stade indivis jusqu'à la fermeture du blastopore. C'est en elle que résident les propriétés d'imperméabilité, de coordination des mouvements cellulaires, de contractilité et d'expansibilité d'ensemble du blastodisque qui entrent en jeu à divers stades de la prémorphogenèse et de la morphogenèse. Vis-à-vis de ce revêtement 'actif' le groupe blastomérien profond fait plutôt figure de masse inerte, ou tout au moins incohérente, incapable d'initiative' et dont les possibilités morphogénétiques, en particulier les mouvements lors de la gastrulation, ont besoin d'être coordonnées, dirigées par la couche enveloppante. Mais c'est de cet ensemble que dérive la quasi-totalité du matériel cellulaire qui édifiera l'embryon; la couche enveloppante ne constituera que l'assise la plus superficielle de l'épiderme, après avoir fourni au matériel de la zone marginale un appoint cellulaire, presque négligeable en quantité.

De la coopération de ces deux constituants résultent la mise en place et la différenciation de l'embryon. Si nous empêchons cette coopération qu'advient-il de la morphogenèse?

Une telle épreuve peut être tentée en débarrassant le blastoderme de sa couche enveloppante et laissant la masse des blastomères profonds adhérente au vitellus. Par suite de l'intervention de mouvements de réaction du syncytium vitellin et de la pellicule périvitelline, les résultats de cette expérience, souvent contradictoires, sont difficiles à interpréter. Il apparaît alors plus simple de faire la même opération sur des blastodermes séparés de leur vitellus; par cette méthode nous avons obtenu quelques résultats qui valent d'être analysés.

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MATÉRIEL ET MÉTHODE

Des œufs de *Salmo irideus* sont placés quelques heures dans une solution très diluée de rouge neutre, ce qui permet de limiter pratiquement la coloration du blastoderme à la seule assise cellulaire de la couche enveloppante. Cette coloration préalable facilite beaucoup la conduite de l'opération. La coque est ensuite ouverte et la couche enveloppante est 'pelée' à l'anse de platine, opération relativement facile sur l'œuf de Truite; avec un peu d'habitude il est possible d'enlever la couche d'une seule pièce en n'entraînant avec elle que peu de blastomères profonds. Couche enveloppante et masse de blastomères profonds détachés du vitellus, sont alors cultivés en godets séparés à la température de 7-8°. Le liquide de culture est le milieu de White (1946) réduit à sa portion minérale et à son glucose et dont la teneur en Ca a été amenée au même taux que dans le liquide de Holtfreter:

Dextrose	0,85 g.
NaCl	0,7 g.
KCl	0,0375 g.
CaCl ₂	0,029 g.
MgSO ₄	0,0275 g.
FeNO ₃	0,14 mg.
Na ₂ HPO ₄	0,0145 g.
KH ₂ PO ₄	0,026 g.
Eau bidistillée q.s.	100 cm. ³

Le grattage de la couche enveloppante peut être effectué aux stades morula à petites cellules, blastula, très jeune gastrula. Passé ce dernier stade le toit blastocélien devient trop mince pour qu'on puisse arracher la couche sans entraîner avec elle les 3 ou 4 assises cellulaires sous-jacentes.

RÉSULTATS

(1) *Blastomères profonds*

Sur 50 cultures effectuées il n'a été obtenu que 6 résultats analysables, tous à partir de jeunes gastrulas. En effet, les explants de blastomères profonds (blastomères grattés) peu cohérents se dissocient souvent et surtout se nécrosent plus rapidement, semble-t-il, que ceux des blastomères complets (c'est-à-dire pourvus de leur couche enveloppante). Déjà la survie et la différenciation des blastodermes complets sont des phénomènes capricieux; bien souvent ces explants se nécrosent ou survivent sans se différencier, sans que nous puissions préciser les raisons d'un tel comportement. Dans le cas des explants grattés où nous multiplions les chances de 'léser' le blastoderme, il n'est pas étonnant que les résultats soient si maigres.

Des explants grattés de morula ou de blastula ont pu survivre parfois dans d'assez bonnes conditions de conservation pendant 5-7 jours, mais se présentent seulement comme des amas informes sans traces de différenciations. Tout au

plus observons-nous des tentatives de groupements cellulaires, séparés du reste de la masse par des limites plus ou moins nettes correspondant à des changements d'orientation des cellules. Le blastocèle se constitue dans les explants de morula. Dans l'ensemble la morphologie est comparable à celle des 'hyperblastulas' (Oppenheimer, 1936) réalisées par les blastodermes complets prélevés très jeunes. Une autre constatation, valable pour tous les explants quel que soit leur stade de prélèvement, qu'ils soient ou non différenciés, est que la couche enveloppante ne se reconstitue pas. Il peut en subsister des fragments qui se reconnaissent facilement sur la surface de l'explant par la forme et l'alignement régulier de leurs



FIG. 1. Coupe d'un explant de blastomères profonds prélevé au stade gastrula jeune et fixé 5 jours après. *c*, corde; *ce*, fragments de couche enveloppante ayant subi l'étalement épibolique; *s*, surface de l'explant formée par les blastomères profonds.

cellules. Dans les explants gastruléens ces îlots de couche enveloppante sont entrés en activité épibolique comme le témoignent l'aplatissement et l'étirement de leurs cellules (Fig. 1). Une telle activité ne se manifeste dans aucun explant prégastruléen alors que des œufs témoins, fixés à la même date que ces explants, sont en pleine épibolie.

Si nous examinons maintenant les quelques explants qui ont subi des différenciations nous noterons d'abord certaines particularités de leur morphologie générale comparée à celle d'explants complets, différenciés, du même âge.

Tandis que l'explant complet se roule, en quelques heures, en une sphère parfaite à surface lisse, par un mouvement dû surtout à l'activité de la couche enveloppante (Devillers, 1951*b*), l'explant gratté ne subit qu'un lent modelage par remaniement et accroissement des contacts intercellulaires et finalement il esquisse vaguement la forme d'une sphère à surface rugueuse dont les cellules superficielles arrondies ne présentent aucune ordonnance régulière et peuvent même se détacher. De tels explants conservent cette forme jusqu'à la fin de leur survie sans jamais subir l'allongement rencontré si souvent avec les blastodermes complets.

La différenciation la plus nette, obtenue avec les jeunes gastrulas, est celle du tissu cordal (Fig. 1), bien reconnaissable à la forme de ses hautes cellules, empilées, écrasées les unes contre les autres, à contenu plus clair que celui des autres cellules. Le degré de différenciation de ce tissu atteint parfois celui de la corde produite par des blastodermes complets mais plus généralement il est moins poussé; l'aspect est plus embryonnaire et les limites de ces fragments de corde sont moins bien tranchées par rapport au reste de l'explant. Du point de vue topographique ce matériel cordal présente deux caractéristiques: d'une part il n'a guère subi d'élongation et d'autre part il se rencontre à l'état de noyaux plus ou moins importants, isolés les uns des autres, soit en pleine masse de l'explant, soit en surface, c'est-à-dire avec une localisation tout à fait anormale.

Les somites sont apparus mais avec une différenciation toujours moins bonne que celle de la corde. On peut en reconnaître des séries, assez réduites en longueur, où la métamérie se distingue et où l'ordonnance des cellules est assez caractéristique.

Les meilleurs s'alignent en courtes séries où la métamérie se manifeste assez bien; le groupement des cellules dans chaque somite y est bien caractéristique. Mais d'autre part, dans la masse de l'explant se rencontrent de nombreuses formations, isolées, qui montrent une disposition radiaire des cellules autour d'une zone centrale, pleine ou creusée d'une petite lumière. Dans certains cas il s'agit incontestablement de somites, mais pour nombre d'entre eux la détermination devient beaucoup plus difficile et douteuse.

En ce qui concerne la topographie nous pouvons, comme pour la corde, observer la dispersion de ces différenciations dans toute la masse et la surface de l'explant. D'autre part elles ne présentent pas les relations typiques avec les formations cordales.

Un troisième groupe de différenciations est constitué par des structures neuroïdes (Fig. 2). Ce sont des masses irrégulières à noyaux arrondis, plutôt groupés à la périphérie et laissant vers le centre une zone de cytoplasme clair. Jamais il n'a été rencontré de formations nerveuses embryonnaires typiques, immédiatement reconnaissables.

Enfin des formations tubulaires ont été observées à plusieurs reprises. Elles sont en général courtes mais dans un cas, un tel tubule, bien organisé, fermé aux deux extrémités, occupe toute la hauteur de l'explant. La lumière centrale y est

bien nette, la paroi composée, sur presque toute sa longueur, d'une seule assise cellulaire. La détermination en est difficile; il pourrait s'agir de formations du type lame latérale.

En surface, les fragments de couche enveloppante subsistant, se sont étirés et présentent les noyaux allongés, caractéristiques de cette structure lorsqu'elle accomplit son mouvement épibolique, mouvement favorisé ici par l'existence d'un substratum.



FIG. 2. Même explant que dans la fig. 1. *n*, masse neuroïde; *som*, différenciation de type somitique.

(2) Couche enveloppante

Très rapidement l'explant de couche enveloppante se roule en une sphère parfaite qui subsiste sous cet état un certain temps, puis, à un moment qui doit correspondre, dans l'histoire de l'œuf normal, à la mise en route de l'épibolie, la forme commence à se modifier; l'explant s'allonge et sa surface se hérissé de plissements (Fig. 3) qui sont le témoignage d'un étalement de la couche enveloppante, correspondant au processus épibolique, incomplètement réalisé ici, par suite de l'absence de substratum sur lequel les cellules puissent adhérer et ramper. Ces dernières conservent alors une forme plus arrondie.

Les coupes ne montrent aucune différenciation dans ces explants, qui pourtant comportent une portion de zone marginale. Peut-être la quantité de ce matériel est-elle trop faible ou bien les cellules constitutives de cette région ont-elles abandonné la couche, réalisant, *in vivo*, le processus d'ingression observé sur l'œuf normal (Devillers, 1951a). Il n'est pas possible d'en décider.

Une observation intéressante concerne les explants de couche enveloppante qui ont entraîné avec eux une petite quantité de matériel profond (Fig. 3). La couche enveloppante forme alors un sac enveloppant complètement un noyau de cellules profondes qui s'est parfaitement différencié en corde, somites, moelle, dans un état comparable aux formations rencontrées dans des blastodermes complets, malgré la faible quantité de matériel cellulaire disponible.

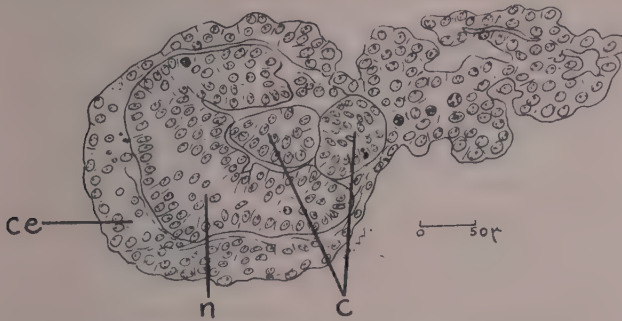


FIG. 3. Coupe d'un explant de couche enveloppante prélevé sur une très jeune gastrula et fixé 6 jours après; il contient dans sa partie gauche un peu de matériel profond. c, corde; ce, couche enveloppante; n, différenciation neurale.

DISCUSSION

Les résultats de ces expériences, bien qu'ils apparaissent assez maigres, permettent cependant de tirer quelques conclusions sur les rôles respectifs des deux constituants du blastoderme dans la mécanique gastruléenne.

Toutes les constatations précédentes soulignent le rôle de premier plan joué par la couche enveloppante dans la vie du blastoderme.

Par sa situation superficielle la couche enveloppante contient le cortex de l'œuf indivis, cortex dont nous savons qu'il présente les mêmes propriétés que celui de l'œuf d'Amphibien. Mais alors que chez l'Amphibien il est possible aux stades cellularisés de détacher le 'coat' des cellules périphériques, une telle opération est impossible chez le Poisson. Nous homologuerons alors tout l'ensemble de la couche enveloppante, héritière cellularisée, du cortex de l'œuf indivis, au moins au 'coat' de l'œuf d'Amphibien. Le cortex d'abord, puis la couche enveloppante, se présentent alors comme une véritable différenciation primaire du blastoderme, différenciation qui, une fois formée, n'est plus capable de régénérer (p. 266); ce n'est donc pas une simple modification superficielle du blastoderme, résultant d'une action du milieu sur la surface protoplasmique; c'est une véritable structure organisée ('champ cortical' de Dalcq et de Pasteels) et pendant longtemps c'est même la seule structure organisée de l'œuf. Comme telle elle doit jouer un rôle essentiel aux premières étapes du développement.

Si nous comparons les différenciations obtenues avec des explants complets ou

avec les groupes de blastomères profonds enfermés dans la couche enveloppante d'une part et celles réalisées par les explants grattés d'autre part nous constatons que le degré de différenciation est plus poussé dans les explants de première catégorie que dans ceux de deuxième espèce.

Ce fait doit être mis en relation avec la présence ou l'absence de couche enveloppante et peut être compris avec l'aide des expériences de Niu & Twitty (1953). Ces auteurs ont observé que des explants de mésoblaste axial d'amphibien, surtout lorsqu'ils ne sont pas protégés par le 'coat', laissent diffuser dans le milieu de culture des substances capables de provoquer la différenciation de fragments ectoblastiques. Une telle diffusion est empêchée, dans l'œuf complet, par le revêtement imperméable du 'coat'.

Or chez le Poisson il est bien connu que le cortex présente ces mêmes propriétés d'imperméabilité relative qui, normalement, interviennent dans la régulation osmotique. Elles doivent jouer aussi lors des cultures de blastodermes complets qui, une fois roulés en boule, sont complètement isolés du milieu par la couche enveloppante. Si nous admettons qu'avant l'explantation le disque embryonnaire a eu le temps d'accumuler à partir du vitellus les réserves nécessaires à l'élaboration de 'substances morphogénétiques',¹ celles-ci seront conservées dans l'explant complet alors qu'en l'absence de revêtement imperméable elles diffuseraient dans le milieu, cette diffusion étant favorisée par le manque de cohésion cellulaire. Donc, dès le début de l'explantation, le germe gratté commence à s'appauvrir. Que se passe-t-il alors à l'époque où devrait débiter la différenciation ?

Les explants prégastruléens n'avaient, d'une part, accumulé que peu de réserve, et d'autre part étaient restés plusieurs jours en contact avec le milieu de culture.² Ils sont donc certainement très pauvres en substances morphogénétiques et leur morphogenèse se limite à des essais, avortés, de ségrégation cellulaire par modification des propriétés de surface; leur différenciation s'arrête à ce tout premier stade d'apparition de l'adhésivité sélective. Dans les explants gastruléens la quantité de substances de réserve était suffisante pour que puisse s'édifier les premiers systèmes enzymatiques, Spiegelman (1948) (?) responsables de l'auto-différenciation cordale. Ce tissu serait alors à considérer comme la différenciation nécessitant le 'pattern' enzymatique le plus simple, 'pattern' qui irait ensuite en se transformant et en se compliquant pour pouvoir provoquer l'apparition des autres systèmes organiques de l'embryon. Un tel perfectionnement du 'pattern' exige un apport continu de matériaux que l'embryon normal puise dans le vitellus. Dans le cas d'un germe explanté, les réserves, limitées, doivent être appauvries, tant en quantité qu'en qualité, pour la genèse de la corde, ce qui

¹ En l'absence de données biochimiques précises nous donnerons à ce terme de 'substances morphogénétiques', volontairement, une acception très vague. Il peut tout aussi bien s'agir de substances directement utilisées pour la production d'énergie, que de systèmes enzymatiques responsables de la différenciation et de la croissance.

² Enfin il leur manque une partie importante du cytoplasme pérblastique qui intervient dans la détermination de la zone marginale (Oppenheimer, 1936; Tung & Tung, 1944; Devillers, 1951b).

n'autorise qu'une esquisse de différenciation somitique et neurectoblastique qui ne dépasse pas le niveau neuroïde. A propos des structures dérivées du neurectoblaste se pose d'ailleurs encore une fois (voir Tung & Tung, 1944; Devillers, 1948b) le problème de leur origine par induction ou par autodifférenciation. Leur contact avec des fragments cordaux, ce qui n'est pas toujours le cas, ne provoque pas d'amélioration de leur degré de différenciation. Il est certain que le système inducteur/réacteur est loin de posséder tout ce que nécessite son fonctionnement normal.

Si nous examinons maintenant le rôle de la couche enveloppante dans les mouvements morphogénétiques, nous trouvons dans nos expériences une confirmation de son intervention décisive, sinon même exclusive, dans le processus d'épibolie du blastoderme. Les explants de couche enveloppante pure présentent une augmentation très nette de leur surface à partir d'une certaine époque, ce qui montre le caractère intrinsèque de cette transformation. Les explants complets s'allongent très généralement, même si leur masse interne ne se différencie pas, tandis que les explants grattés ne subissent aucun allongement. Dans le développement normal les matériaux constitutifs de l'embryon sont donc passivement étalés par les mouvements de la couche enveloppante, l'élongation de la corde ne jouant qu'un rôle accessoire dans l'étirement de l'embryon (Devillers, 1948b).

Reste la question des mouvements d'invagination. Nous ne savons pas grand-chose de leur déterminisme. Tout au plus connaissons-nous l'existence des mouvements d'ingression du matériel cellulaire de la couche enveloppante appartenant à la zone marginale (Devillers, 1951a). Mais il s'agit là d'un apport cellulaire réduit dont le mouvement joue peut-être un rôle dans l'invagination générale en dirigeant la course du matériel profond (?). Mais il n'y a pas de mouvement d'invagination d'ensemble du cortex comme cela s'observe chez les Amphibiens (Holtfreter, 1943). C'est essentiellement le matériel profond qui est intéressé par l'invagination et l'on pourrait penser que la présence d'un support solide, adhésif, constitué par la surface du syncytium vitellin est nécessaire à sa réalisation. Qu'advient-il alors des mouvements d'invagination dans les explants séparés du vitellus?

Luther (1936) (greffes en coelome vitellin de blastodermes de *Salmo*) et Tung & Tung (1944) (sur *Carassius*) admettent, qu'au moins dans certains cas, la mise en place des matériaux n'a pas eu lieu et qu'ils se sont différenciés *in situ*.

Pour Oppenheimer (1936), les explants de *Fundulus* ont subi une invagination normale ou subnormale et c'est certainement ce qui se produit aussi dans les explants complets de *Salmo* cultivés *in vitro*: la corde y est toujours en position profonde, détachée de la surface, comme le sont aussi les somites, et malgré les bouleversements topographiques observés dans les embryons ainsi obtenus, les relations mutuelles des diverses structures axiales sont proches de la normale.

Dans nos explants grattés, au contraire, nous trouvons fréquemment la corde et les somites en surface, tout comme dans certains embryons figurés par

Tung & Tung (voir fig. 14 de leur mémoire de 1944). Par comparaison avec la topographie réalisée par les explants complets, il est certain que dans l'explant gratté l'invagination n'a pas eu lieu et que les ébauches se sont différenciées *in situ*, après avoir subi une désorganisation due aux remaniements cellulaires.

Si le syncytium vitellin joue peut-être un rôle dans la gastrulation normale (?) ce rôle n'est certainement pas décisif (voir aussi Oppenheimer, 1936) puisque les explants complets, roulés en boule, subissent les mouvements d'invagination dans des conditions différentes, plutôt comparables à celles réalisées dans un œuf d'Amphibien où les cellules de la zone marginale, et de l'endoderme, glissent les unes sur les autres sans l'intervention d'un support immobile.

D'autre part nous aboutissons à ce résultat paradoxal qu'en l'absence de couche enveloppante l'invagination ne se produit pas alors que cette structure ne paraît pas intervenir directement dans ce mouvement au cours du développement normal. Faut-il faire intervenir la couche enveloppante comme élément coordinateur des mouvements individuels des blastomères profonds, mouvements qui peut-être subsisteraient partiellement dans les explants grattés, puisque des noyaux de corde sont en position centrale, mais se développeraient de manière incohérente? Faut-il voir une liaison entre cet affaiblissement extrême des mouvements d'invagination et la diffusion des substances morphogénétiques? Nous ne pouvons actuellement donner de réponse à cette question.

RÉSUMÉ

Des disques embryonnaires de *S. irideus*, séparés de leur vitellus et dépouillés de leur couche enveloppante, ont été cultivés en milieu synthétiques. Ils ont différencié de la corde, des somites et des formations neuroïdes.

Les différenciations obtenues sont beaucoup moins parfaites que celles obtenues dans les mêmes conditions avec des blastodermes pourvus de leur couche enveloppante. Cette différence dans l'état de développement est attribué à la diffusion, dans le milieu de culture, de 'substances morphogénétiques' élaborées par le disque embryonnaire. Une telle diffusion ne peut se produire lorsque le blastoderme est pourvu de sa couche enveloppante, imperméable.

D'autre part l'étude de la localisation des différenciations montre que les ébauches présomptives de la gastrula se sont différenciées *in situ* sans subir l'invagination alors que ce mouvement se produit dans les explants pourvus de couche enveloppante.

Le rôle de la couche enveloppante dans la réalisation du mouvement d'épibolie est mis en évidence par ces expériences et par des cultures *in vitro* de couche enveloppante séparées de la masse des blastomères sous-jacents.

SUMMARY

Blastoderms of *Salmo irideus*, separated from their yolk and with their covering layer (*Deckschicht*) removed, were cultured in a synthetic medium. Notochord, somites, and neural structures differentiated.

The differentiation obtained was much less perfect than that obtained in the same conditions in blastoderms provided with their covering layer. This difference in the degree of development is attributed to the diffusion into the culture medium of 'morphogenetic substances' elaborated by the embryonic disc. Such diffusion cannot occur when the blastoderm is provided with its impermeable covering layer.

A study of the localization of the differentiations shows that the presumptive rudiments of the gastrula have differentiated *in situ* without undergoing invagination, although this movement takes place in explants possessing their covering layer.

The role of the covering layer in the occurrence of epibolic movement is demonstrated by these experiments, and by *in vitro* cultures of the covering layer alone, without the mass of underlying blastomeres.

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The Effect of Unilateral Blockage of a Pronephric Duct upon the Development of the Pronephroi in *Triturus helveticus*

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WITH ONE PLATE

INTRODUCTION

WHEN one member of a pair of pronephroi is removed at an early larval stage in *Ambystoma* (*Siredon*) and *Triton cristatus*, the remaining one becomes larger than normal during subsequent development (Howland, 1916, 1921; Fox, 1956). The question may be posed as to whether pronephroi exercise mutual inhibitory effects upon one another, so that the removal of one of the pair allows the remaining organ to increase in size. In *Rana nigromaculata* and *Bufo vulgaris japonicus* partial or total reduction of one pronephric duct leads to hypertrophy of the contra-lateral pronephros and duct (Miura, 1930). No numerical data were supplied in this investigation. Obstruction to a Wolffian duct in the embryo chick leads to hydronephrosis on the same side and the other mesonephros becomes approximately twice the normal size (Boyden, 1927). Similarly, the unoperated kidney of an adult shows compensatory hypertrophy when compared with the normal after ureteral ligature and hydronephrosis of its fellow, in the hen (Likhatscheff, 1896), albino rat (Arataki, 1927), rabbit (Haseyama, 1931), and man (Perl, 1872; Haymans, 1942). Further, the cells of both kidneys show an increase in the rate of mitosis after unilateral ureteral ligature in the adult rat when compared with controls. The increase is greater in the ligatured kidney, and after 4 days the other one attains a level comparable to that shown by a compensating pronephros after unilateral nephrectomy (Herlant, 1948). It would seem that if a functional kidney enlarges under these conditions, then its non-functional blocked partner does not exercise any inhibition on this development.

The present work describes quantitatively the changes which take place in both pronephroi of a newt larva when one of the pair is prevented from functioning normally by blocking its duct. The results show that both pronephroi

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significantly increase in size when compared with controls during subsequent development.

MATERIAL AND METHODS

Two groups of eggs of *Triton helveticus* at stages 21–23 (Glaesner, 1925) were used in the investigation. Members of the operated group were decapsulated in full strength Holtfreter solution and, with a sterile glass needle and hair loop (Hamburger, 1950), a short section of tissue containing the presumptive pronephric duct cells was removed on one side. The region where the duct was thus sectioned lay just behind the pronephric blastema, between the somites and the ventral yolk sac. The actual duct cells cannot be distinguished from the surrounding tissue at this stage, but the success of the removal can be determined when the larvae are sectioned at a later stage. The eggs were allowed to remain in full strength Holtfreter solution for 6 hours to facilitate healing and they were then transferred to crystallizing dishes, four to each dish, containing approximately 75 c.c. of one-tenth Holtfreter solution with 0.2 per cent. sodium sulphadiazine added (Fox, 1955). The temperature varied between 18° and 20° C. The base of each dish was covered with a thin layer of gelatine and about 1 inch of air space lay between the surface of the solution and the glass dish cover. Solutions were changed every 24 hours and small *Daphnia* were added to the medium 12 days after operation. Members of the control group were treated in an identical manner except that merely a surface wound was made in the same region and the pronephric duct cells were not disturbed. Specimens were killed in Smith's fixative 20 days after operation. Seven larvae of the control group and ten of the operated group, all chosen at random from the total number of fixed specimens, were used in the analysis. Specimens were blocked in paraffin wax, sectioned transversely at 10 μ , and stained with Ehrlich's haematoxylin and aqueous eosin. One further specimen in each group was sectioned horizontally.

In order to obtain the various measurements, sampling methods and measuring techniques identical to those in previous investigations were used (Fox, 1955, 1956). These techniques include the use of direct counting of the nuclear population of the pronephros from serial sections. The mean antero-posterior length of a pronephros nucleus, at right angles to the transverse section, was obtained from the horizontal sections and was used to apply a correction to the crude nuclear counts (Abercrombie, 1946). Microprojection at known magnification, measurement of the areas of the various components of the pronephric system with a planimeter, and measurement of the length as seen in section of the inside of the tubule wall and of the external boundary of the glomus with a map measurer, provided the required volumes and surface areas of these components. The overall volume of the pronephros is the sum of the total volume of the cells and of the tubule lumina. As the pronephros cells are practically all uninucleate, the calculated cell volume was obtained by dividing the nuclear population into

the total cell volume. Nose-to-cloacal body length, antero-posterior lengths of the pronephros and of the glomus, and the diameter of the base of the nephrostomial tubule (where the latter opens into the coelom) were obtained by summing the transverse sections.

The unblocked pronephros of the operated group will be designated the functional pronephros and the other the blocked pronephros. Control pronephroi are provided by members of the control group.

RESULTS

There is no significant difference in mean total length and nose-to-cloacal length between specimens of the control and operated groups fixed 20 days after operation. Larvae from both groups were indistinguishable in external form.

Comparison between control pronephroi and functional pronephroi (Plate, figs. A, B, C)

The components of the functional pronephroi show significantly higher values than those of the controls for all the measurements made, except for the mean

TABLE 1

Various measurements of control and operated specimens of Triturus larva, fixed at comparable stages

	Controls	Operated Specimens	
		Functional pronephroi	Blocked pronephroi
Number of specimens	7	10	9
Number of pronephroi	14	10	9
Mean total length of specimens (approx. mm.)	9.6 ±0.170	9.8 ±0.170	9.7 ±0.169
Mean nose-to-cloacal length (mm.)	5.18 ±0.104	5.29 ±0.087	5.26 ±0.093
Mean antero-posterior length of pronephros (mm.)	0.346 ±0.009	0.416 ±0.013	0.394 ±0.025
Mean nuclear population of pronephros	466 ±19	658 ±29	778 ±49
Mean total volume of cells of pronephros (mm. ³ × 10 ³)	2.3 ±0.11	4.1 ±0.28	3.5 ±0.28
Mean total volume of lumina of tubules (mm. ³ × 10 ³)	1.1 ±0.10	2.5 ±0.29	3.4 ±0.80
Mean overall volume of pronephros (mm. ³ × 10 ³)	3.4 ±0.18	6.6 ±0.51	6.9 ±1.07
Mean area of internal surface of tubules (mm. ²)	0.1793 ±0.0101	0.2930 ±0.0255	0.2664 ±0.0273
Mean volume of individual cell of pronephros (μ ³)	5038 ±177	6145 ±254	4488 ±183
Mean antero-posterior length of 240 pronephric nuclei in horizontal section (μ)	12.255 ±0.182	12.198 ±0.171	12.103 ±0.205

antero-posterior length of the anterior nephrostomial tubule base (Tables 1, 2). Percentage superiority of the means of the functional pronephroi over the con-

TABLE 2

Various measurements of the nephrostomial canals of control and operated specimens of Triturus larva, fixed at comparable stages

	Controls	Operated specimens	
		Functional pronephroi	Blocked pronephroi
Number of specimens	7	8	9
Mean total length of specimens (approx. mm.)	9.60 ±0.170	9.87 ±0.183	9.70 ±0.169
Mean nose-to-cloacal length of specimens (mm.)	5.18 ±0.104	5.35 ±0.077	5.26 ±0.093
Number of pronephroi	12	8	9
Number of nephrostomes	24	16	18
Diameter of nephrostome base (including walls of anterior and posterior nephrostome together (mm.))	0.0496 ±0.0026	0.0594 ±0.0031	0.0661 ±0.0058
Diameter of anterior nephrostome base (mm.)	0.0583 ±0.0027	0.0650 ±0.0038	0.0789 ±0.0079
Diameter of posterior nephrostome base (mm.)	0.0417 ±0.0024	0.0537 ±0.0042	0.0533 ±0.0062

TABLE 3

Various measurements of the glomi of controls and operated specimens of Triturus larva, fixed at comparable stages

	Controls	Operated specimens	
		Functional pronephroi	Blocked pronephroi
Number of specimens	7	10	10
Mean total length of specimens (approx. mm.)	9.6 ±0.170	9.8 ±0.170	9.8 ±0.170
Mean nose-to-cloacal length of specimens (mm.)	5.18 ±0.104	5.29 ±0.087	5.29 ±0.087
Number of glomi	14	10	10
Mean antero-posterior length of glomus (mm.)	0.201 ±0.0065	0.214 ±0.0118	0.213 ±0.0084
Mean total volume of glomus (mm. ³ × 10 ³)	0.49 ±0.021	0.58 ±0.026	0.60 ±0.035
Mean total external surface area of glomus (mm. ²)	0.0391 ±0.0014	0.0441 ±0.0022	0.0438 ±0.0022

trols are as follows: antero-posterior length of pronephros, 20; nuclear populations, 41; total volume of cells, 78; total volume of tubule lumina, 127; overall volume of the pronephros, 94; internal surface area of the tubules, 63; calculated

individual pronephric cell volume, 22; antero-posterior length of the posterior nephrostomial tubule base, 28; antero-posterior length of the anterior and posterior nephrostomial tubule bases together, 19. The enlarged functional pronephroi also differed from the control pronephroi in having a duct greatly expanded along its whole length (Plate, figs. D, F).

The mean total volume of the glomus adjacent to the functional pronephros is significantly larger than that of the control glomi by 18.4 per cent. The higher mean values of the antero-posterior length and total external surface area of the glomus were not significant (Table 3).

Two out of 14 control pronephroi and 2 out of 10 functional pronephroi differed from the rest in having only one large nephrostomial canal opening into the coelom from the tubular mass, instead of two (Field, 1891). Apart from being uneinephrostomial these pronephroi did not differ in any way from the other members of their group. The antero-posterior lengths of the bases of these nephrostomial canals were 0.08, 0.10, 0.09, and 0.10 mm. respectively. In one control pronephros the anterior and posterior nephrostomes were separated by merely 20–30 μ , whereas in the other pronephroi the two nephrostomes are widely separated. This would suggest that the single nephrostomial canals are really anterior and posterior ones which have fused at their bases, opening into the coelom by a common canal. These single canals have been omitted from the quantitative analysis so that only 24 canals from 12 control pronephroi and 16 from 8 functional pronephroi were available (Table 2).

Comparison between control pronephroi and blocked pronephroi (Plate, figs. A, B, C)

Owing to damage to one during preparation only nine blocked pronephroi were analysed. The glomus adjacent to this damaged pronephros was, however, undamaged and was included in the analysis.

The blocked pronephroi show significantly greater mean values than the controls in all measurements made (Tables 1, 2), except that of the antero-posterior length of the posterior nephrostomial tubule base which is not significantly different, and the mean calculated individual cell volume which is significantly reduced by 11 per cent. The percentage superiority of each of the means of the blocked pronephroi over the controls is: antero-posterior length of the pronephros, 14; nuclear population, 67; total volume of cells, 52; total volume of tubule lumina, 209; overall volume of the pronephros, 103; internal surface area of the tubules, 49; antero-posterior length of the anterior nephrostomial tubule base, 35; and the anterior and posterior nephrostomial tubule bases together, 33.

The mean total volume of the glomus adjacent to the blocked pronephros is significantly larger than that of the control glomi by 22 per cent. As in the case of the glomus adjacent to the functional pronephros, increases in the mean antero-posterior length and total external surface area were not significant (Table 3).

The mean antero-posterior length of the blockage to the pronephric duct in the nine members of the operated group was 0.46 ± 0.060 mm. At the site of the operation there was a total absence of the duct (Plate, fig. E), and behind this a degenerate strand of cells without a lumen. In most cases the blocked inflated pronephric duct became sealed off as an expanded blind end. Behind the operated region the duct has a small flattened lumen (Plate, fig. F). The distal region of the duct posterior to the lesion is identical with a duct after its pronephros has been extirpated (Fox, 1956).

Comparison between functional pronephroi and blocked pronephroi of operated animals

There are no significant differences between the mean values of the various components of the pronephroi and adjacent glomi in the two groups, except for the significantly lower value of the mean nuclear population (15 per cent.) and the significantly higher value of the mean calculated individual cell volume (37 per cent.) of the functional pronephroi. In other words the functional pronephros possesses fewer cells, but approximately the same total volume of tissue as the blocked pronephros (Tables 1, 2, 3). The glomi adjacent to either the functional or the blocked pronephroi show a similar significant enlargement of mean total volume when compared with controls. The glomerular system thus responds as a whole to the changes which take place in the pronephroi (blocked or unblocked) of the operated animals.

DISCUSSION

A previous analysis of compensation in the remaining pronephros of *Triturus cristatus carnifex* larvae after unilateral pronephrectomy, showed significant increases in various measurements of the pronephroi, compared with unoperated controls (Fox, 1956). The extent of these enlargements in relation to unoperated controls may be compared with those found in the present work for the remaining functional pronephros of *T. helveticus* in which one of the pronephric ducts has been blocked. Expressed as a percentage of the control values, the increases in various measurements are as follows (those of *T. helveticus* are shown in brackets). Lumen volume, 100 (127); overall volume, 52 (94); internal surface area of tubules, 49 (63); total cell volume, 34 (78); nuclear population, 17 (41); calculated individual cell volume, 15 (22); antero-posterior length of pronephros, 14 (20). There is, therefore, a similar pattern of compensatory response. The rank order of percentage increases is the same except for the reversed position of the internal surface area and total cell volume. Relative increases are greater in *T. helveticus* probably because these specimens survived 20 instead of 13 days after operation. If paired pronephroi produced substances which inhibited or in some way regulated each other's growth, then a functional pronephros in the presence of its non-functional partner would not compensate. As compensation

in this case is demonstrated in *Triturus* it follows that inhibition by one pronephros upon its fellow is unlikely..

The fact that a functional pronephros compensates notwithstanding the presence of its blocked undamaged partner does not support the theory of auto-regulation of growth by homologous organs (Weiss, 1952, 1953, 1955). Some damage to the presumptive duct cells behind the blocked pronephros does occur, but this effect is limited in space and time. The pronephros cells themselves were undisturbed and though the tubules were stretched, no damage to them was apparent.

However, local tension to an organ may stimulate mitotic activity (see Abercrombie, 1957). Blocked pronephric tubules are under tension greater than normal, resulting in an inflated condition (Holtfreter, 1944). This is probably true in the case of the functional pronephros also and the nephrostomes and nephrostomial canals are enlarged in both cases. This increase in intra-tubular pressure in the blocked pronephros is presumably brought about by the activity of the nephrostomial cilia continuing to drive coelomic fluid into the closed system. Increase in tension may also be an important factor in expanding the pronephric duct of the functional pronephros of the operated group, like the remaining duct of an anuran larva which has suffered unilateral duct extirpation (Miura, 1930), and the duct of a remaining pronephros in a unilaterally pronephrectomized *T. cristatus* larva (Fox, 1956).

SUMMARY

1. Larvae of *Triton helveticus*, 20 days after the pronephric duct on one side has been blocked at the caudal bud stage, do not differ from controls in total length or nose-to-cloacal length.

2. The unblocked functional pronephros of the other side shows significantly higher mean values for the following measurements when compared with control pronephroi in sham-operated animals: antero-posterior length, nuclear population, total volume of cells, total volume of lumina, overall volume of the pronephros, internal surface area of tubules, calculated individual cell volume, and antero-posterior length of the posterior nephrostome base.

3. The blocked pronephros also shows significantly higher values than controls in all these measurements, except that it is the anterior nephrostome base and not the posterior which significantly increases in antero-posterior length, and that the calculated individual cell volume significantly falls.

4. The glomus adjacent to the functional pronephros and that adjacent to the blocked pronephros do not differ significantly in size and shape. Both groups show a significant superiority in mean total volume over the controls.

5. The pronephric duct of the functional pronephros, when its partner is blocked, is expanded along its length, like the duct of the remaining pronephros when a larva has undergone unilateral pronephrectomy. The remainder of the

duct behind the lesion of the blocked pronephros is degenerate, and has a flattened reduced lumen like the duct remaining when its ipsilateral pronephros has been extirpated.

6. There is no evidence that a blocked pronephros *in situ* exercises any inhibition on growth of its functional partner. In compensatory growth local intra-tubular tension may be an important influence.

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EXPLANATION OF PLATE

All sections are transverse and 10 μ thick. *Abbreviations:* *bl. dt.*, duct of blocked pronephros; *bl. tub.*, tubule of blocked pronephros; *co.*, coelom; *comp. dt.*, duct of compensated pronephros; *comp. tub.*, tubule of compensated pronephros; *cont. dt.*, duct of control pronephros; *cont. tub.*, tubule of control pronephros; *d.a.*, dorsal aorta; *exp. neph.*, expanded nephrostome; *exp. neph. tub.*, expanded nephrostomial tubule of compensated pronephros; *g.*, gut; *glom.*, glomus; *n.c.*, nerve cord; *neph. tub.*, nephrostomial tubule of control pronephros; *not.*, notochord; *som.*, somite.

FIG. A. Specimen of control group. Section 1.89 mm. from anterior tip of head. Left-hand side pronephros 0.32 mm. and right 0.35 mm. long. Left pronephros 0.05 mm. and right 0.04 mm. from anterior end of pronephros. Specimen 9.5 mm. long, nose-to-cloaca 5.13 mm.

FIG. B. Specimen of operated group. Section 1.88 mm. from anterior tip of head. Left-hand side (functional pronephros) 0.33 mm. and right (blocked pronephros) 0.44 mm. long. Left pronephros 0.20 mm. and right 0.23 mm. from anterior end of pronephros. Specimen 9.5 mm. long; nose-to-cloaca 4.99 mm.

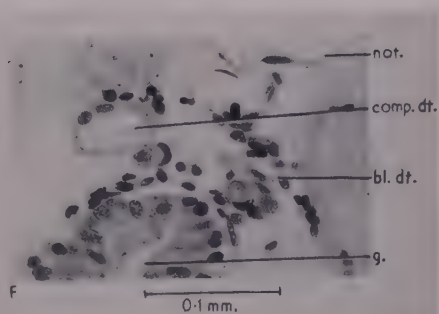
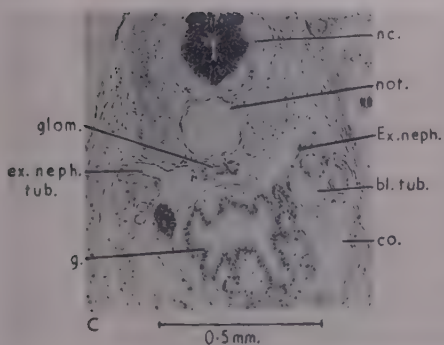
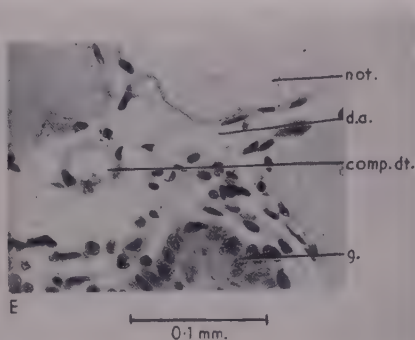
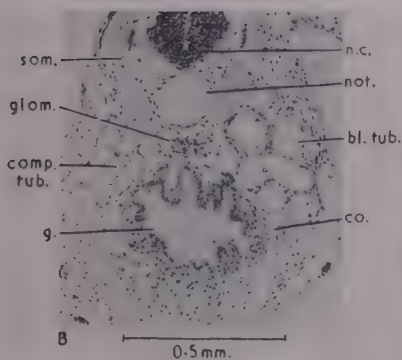
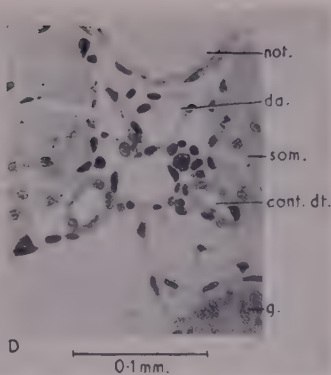
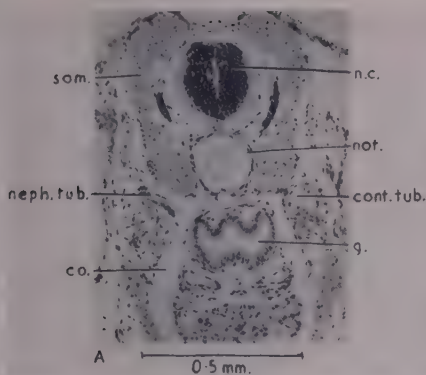
FIG. C. Specimen of operated group. Section 2.04 mm. from anterior end of head. Left-hand side (functional pronephros) 0.41 mm. and right (blocked pronephros) 0.34 mm. long. Left pronephros 0.17 mm. and right 0.09 mm. from anterior end of pronephros. Specimen 10.5 mm. long; nose-to-cloaca 5.54 mm.

FIG. D. Same specimen as Fig. A. Section 3.34 mm. from anterior tip of head.

FIG. E. Same specimen as Fig. B. Section 2.76 mm. from anterior tip of head.

FIG. F. Same specimen as Fig. B. Section 3.41 mm. from anterior tip of head.

(Manuscript received 18:x:56)



H. FOX
Plate

The Morphogenetic Influence of Innervation on the Ontogenetic Development of Muscle-spindles

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WITH TWO PLATES

INTRODUCTION

THE influence of innervation on the differentiation of peripheral structures during ontogenesis has been studied mainly in skeletal muscle.

It has been shown in a number of experimental studies on amphibians that the morphological differentiation of the whole extremity as well as its muscular tissue takes place normally after removal of the medullary plate at the gastrula or neurula stage, although the limb developing without innervation is smaller and its musculature is atrophic (Harrison, 1903, 1904; Hooker, 1911; Hamburger, 1928). In the chick transplantation of the limb-bud into the coelom cavity or on to the chorioallantois leads in the first phase to muscular differentiation despite the fact that the limb is not innervated (Hunt, 1932; Hamburger & Waugh, 1940; Eastlick, 1943; Eastlick & Wortham, 1947). Degeneration and sarcolysis follow the initial differentiation at a very early stage.

Similarly, an histological analysis of pathological material from human and animal embryos indicates that the differentiation of non-innervated muscular tissue takes place; the developing musculature, however, succumbs at a very early stage to degeneration and is practically replaced by adipose tissue (Weber, 1851; Anders, 1921).

Morphological differentiation of muscle-fibres in amphibians evidently takes place independently of the nervous system and may attain maturity, while in birds and mammals only the initial phase of the development of myoblasts and myotubes is possible. Further differentiation is not realized on account of rapid degeneration. An increased dependence of the histogenesis of musculature on the innervation is to be observed in these phylogenetically higher forms. The way in which the influence of the nervous system on morphogenesis of muscle changes during ontogenesis is not, however, known. This problem might be

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elucidated by denervation studies during the later stages of muscular development.

It is possible to recognize three stages in the morphogenesis of muscular tissue: the myoblastic stage, the myotubal stage, and the stage of the mature muscular fibres (Tello, 1922; Studitskij & Striganova, 1951). The myoblastic stage is characterized by the production of elongated pointed myoblasts with oval nuclei from non-differentiated mesodermal cells. The fusion of myoblasts, together with the proliferation of nuclei and the differentiation of myofibrils at the periphery, characterize the myotubal stage. Further stages of development continue in either of two ways. In the majority of myotubes the nuclei migrate to the periphery, myofibrils fill up the space inside the myotube, and a mature muscle-fibre develops. A different course of histogenesis is observed in a smaller number of myotubes which become a part of neuromuscular spindles: nuclei do not migrate towards the fibre surface, but divide intensively, forming a nuclear bag which is a characteristic structure of the intrafusal muscle-fibres, i.e. fibres of the muscle-spindle.

The normal development of a muscle-spindle and its components—intrafusal fibres, nerve-endings, and connective tissue capsule—has been described in birds (Tello, 1922) and in several mammals (Sutton, 1915; Cuajunco, 1927 *a, b*; Kalugina, 1956). There is a marked temporal coincidence between the appearance of primitive nerve-endings and the nuclear proliferation in muscle-fibres surrounded by this network. It is not at all clear, however, whether and to what extent the nervous system influences the specific differentiation of intrafusal fibres.

In this report the development of intrafusal muscle-fibres and their connective tissue capsule was studied in normal and denervated muscle with the aim of elucidating the question whether it is dependent on the influence of the nervous system, or whether the differentiation can take place even at a relatively late stage of development independently of innervation.

MATERIAL AND METHODS

Experiments were performed on rat and rabbit foetuses and on new-born and 20-day-old rats. The stage of differentiation of muscle-spindles in rats, rabbits, and guinea-pigs at birth was determined in a preliminary study. The majority of experiments was done on the rat because muscle-spindles begin to develop very late in this animal and an intra-uterine operation is more easily performed shortly before birth. The development of normal muscle-spindles was observed in the rat from the 17th day of intra-uterine life, i.e. 5 days before birth, till the 30th day after birth in calf-muscles of the hind extremity. Muscles were impregnated in frozen sections (Gross-Bielschovsky) and *in toto* (Cajal V). After embedding in celloidin-paraffin (Péterfi's technique), partial series of calf-muscles were obtained, stained with Harris's haematoxylin-eosin, Heidenhain's haematoxylin, Azan, and a combination of the last two methods.

The development of muscle-spindles has been determined in muscles of the thorax and the forelimb of rats (Kalugina, 1956). The course of spindle development in the calf-muscles is described in detail for two reasons: (1) a slower course was found than the spindles of the thorax, (2) Kalugina primarily observed the development of innervation; a description of cytological details seems to be more important if comparison between the development of muscle-spindles in normal and denervated tissue is to be made.

The sciatic nerve was sectioned in the experimental series in the middle of the thigh in rat foetuses on the 19th–20th day of intra-uterine life, i.e. 60–72 hours before birth, on the first day after birth, and in 20-day-old rats. Material was taken 3, 4, 5, 7, and 10 days after the intra-uterine operation, and after 5 and 10 days in the remaining two series. In rabbit foetuses the sciatic nerve was sectioned on the 21st day of intra-uterine development, i.e. 7 days before birth, but experimental material was obtained only in two cases.

Neurotomy in foetuses was performed under ether anaesthesia of the mother on the exact date after insemination. After laparotomy the uterus was exposed and a purse string suture, which included the foetal membranes, was made above the right hind limb of the foetus (usually in the horn of the uterus). Under microscope control the wall of the uterus was incised and the hind limb exposed. The sciatic nerve was cut and a part was excised in order to prevent regeneration. Bleeding was stopped with the aid of fibrin foam. The wound and the foetal membranes were sutured in the rabbit foetuses only. The exposed limb was slipped back into the uterus and the purse-string suture tightened. The procedure was performed on 2–3 foetuses in each mother. Following this operation the rats were usually able to deliver normally and suckle young.

In the new-born and 20-day-old rats *n. ischiadicus* was sectioned close to the hip-joint and a ligature was applied to the central stump.

RESULTS

A. Development of neuromuscular spindles in the calf-muscles of the rat

On the 17th and 18th day of intra-uterine development a nervous network without terminal endings is present in the calf-muscles of the foetus. Myotubes with indistinct cross-striation do not yet exhibit any individual differences (Plate 2, fig. D). By the 19th–20th day there are to be seen in some of the myotubes zones of densely packed nuclei in the vicinity of nerve-branching arranged along the longitudinal axis of the myotube. Elongated nuclei are placed in rows closer together than in the normal myotube. This represents the beginning of spindle differentiation. The formation of spindles does not, of course, commence abruptly and does not progress quite uniformly even in the same muscle.

Immediately before birth there is further nuclear division in the future equatorial zone of the spindle. In the vicinity of this zone fibroblasts appear in the spaces in-between muscle-fibres. In most cases nerve-fibres advance towards

the future polar region of the spindle, adopt a parallel course along one or both sides of the equatorial zone, and give off fine branches to it.

The first day after birth the equatorial zone in the rat is clearly differentiated and is approximately $40\ \mu$ long and $15\ \mu$ wide, including the differentiating capsule. On longitudinal section 1–2 intrafusal fibres with a course along the axis of the spindle may be observed. The fibres in the equatorial zone are packed with nuclei, which are strongly basophil, mostly polygonal though sometimes oval, and placed in one or two rows close to each other (Plate 2, fig. E). The intrafusal fibres are dilated in the equatorial zone by this accumulation of nuclei, which quite displaces the protoplasm. A capsule of connective tissue containing fibroblasts, placed closely one after the other, is formed around the intrafusal fibres. It is possible to observe fine reticular fibrils, stained by aniline blue, as far as the polar zones. Nerve-fibres form a simple plexus around the intrafusal fibre.

On the 5th day after birth (Plate 1, fig. A) a lymphatic space about $10\ \mu$ wide begins to develop between the capsule and the equatorial zone of the spindle. Most spindles are as much as $500\ \mu$ long and about $30\ \mu$ wide in the equatorial zone. The nuclei in the equatorial zone gradually become less basophil, lighter, and poor in chromatin. In the myotubal zone the nuclei, rounded at the ends, are placed one behind the other in the centre of the muscle-fibre and surrounded by protoplasm containing striated myofibrils. In the distal polar zone the nuclei are placed at the periphery and the muscle-fibres differ from extrafusal fibres only by being of smaller diameter. The capsule of connective tissue is already differentiated in the polar zone of the spindle. Between the 5th–10th day the muscle-spindle acquires its motor innervation. The motor-fibres form primitive end-plates in the polar zone, while the proprioceptive fibres run freely along the intrafusal fibres and produce a plexus with wide loops.

Till the 10th day the diameter of the intrafusal fibre increases mainly in the equatorial zone. The periaxial lymphatic space also widens, so that the spindle reaches $40\ \mu$ in width at the equator (Plate 1, fig. B).

By the 15th day after birth the lymphatic space is already formed in the distal polar zone, but this remains of capillary dimensions. There is no basic change at the equatorial zone, which is generally about $40\ \mu$ on cross-section and about $80\ \mu$ long. Proprioceptive fibres form loose plexi and also accompany the intrafusal fibres, with a wave-like course. It is not yet possible to differentiate between annulospiral and flower-spray endings.

By the 25th day after birth longitudinal growth of the spindles has taken place, so that they have become almost 1 mm. long, though neither the length of the equatorial zone nor the width of the intrafusal fibres has changed significantly. The equatorial zone at this time is $60\ \mu$ long, the myotubal zones are 60 – $100\ \mu$ long, the rest being the polar zones. The lymphatic space widens, measuring $16\ \mu$ at the most, so that the total spindle diameter increases to 50 – $60\ \mu$. The annulospiral and flower-spray endings are being formed (Plate 1, fig. C), and the spindle has all the morphological characteristics of a mature neuromuscular receptor.

B. The morphogenesis of intrafusal fibres in the denervated calf-muscle

1. Section of the sciatic nerve performed in rat fetuses on the 19th–20th day of intra-uterine development

At this time the differentiation of intrafusal fibres has begun by the division of nuclei in the myotubes in the neighbourhood of the branching of proprioceptive nerve-fibres.

Three days after the denervation (4 animals) spindles containing 1–2 intrafusal fibres in the equatorial zone, which may already be 60 μ long, are to be found in new-born rats in sections of the calf-muscles normally innervated. Similar structures do not occur in the denervated extremity. Occasionally muscle-fibres of myotubal character were observed, containing either long nuclei with a fine chromatin structure, or fibres with elongated nuclei arranged closely end-to-end (Plate 2, fig. F), often locally basophil to different degrees. Some of these nuclei are pycnotic with a tendency to karyorrhexis. Occasionally oedematous fibres occur in the musculature. Relatively often very fine fibres, mostly without myofibrillar structure, with elongated nuclei filling up the whole cross-section and placed at considerable distance from each other, may be found.

Very occasionally karyorrhexis was observed in oedematous muscle-fibres. In some of the muscle-fibres an uninterrupted chain of 5–6 tubular nuclei is present either darkly or lightly stained (Plate 2, fig. G). In the lighter nuclei the nuclear membrane at some places disappears and a strand of feebly stained karyoplasm remains in the muscle-fibre and is continued by basophil nuclei about 2 μ wide, surrounded by sarcoplasm, at both ends. All these structures occur quite exceptionally.

Four days after intra-uterine neurotomy (3 animals) thin basophil nuclear chains may be observed in exceptional cases, with overlapping, badly defined boundaries. These nuclei are not always surrounded by sarcoplasm. At both poles, however, there are remnants of sarcoplasm in the form of eosinophil threadlike endings. In one case a similar structure with disintegrating pycnotic nuclei was observed and these were joined together by a fine basophil bridge, which was evidently a remainder of the nuclei of the central zone.

Five days after intra-uterine neurotomy (3 animals) muscle-fibres containing elongated nuclei placed medially end-to-end are rarely to be found in the denervated musculature. In some of these the sarcoplasm is almost non-existent. The structures described occur very rarely—one in 4 or 5 longitudinal sections—while on the control side it is possible to count 25–30 muscle-spindles in the same number of sections.

Seven to ten days after intra-uterine neurotomy (5 animals) simple atrophy takes place in the denervated musculature and disintegration of individual muscle-fibres is rarely to be seen. In sharp contrast stands the musculature of the normal limb, where muscle-spindles are as long as 200 μ .

2. Section of *n. ischiadicus* in rabbit foeti 7 days before birth

The musculature at this time (i.e. the 21st day of intra-uterine development) is at the myotubal stage. The nuclei are placed centrally and the myofibrils at the circumference of the myotube. In some of these myotubes there are 5–6 nuclei arranged end-to-end. This nuclear chain, which is considered to be the initial stage of differentiation of the intrafusal fibre, occurs sometimes parallel in two neighbouring myotubes (see Plate 2, fig. H).

In new-born rabbits the neuromuscular spindles in normal muscles are in an advanced stage of development (see Plate 2, fig. I). The equatorial zone, containing cylindrical nuclei which fill up the whole lumen of the fibre, is 100 μ long, and the myotubal and polar zones can easily be recognized. The connective tissue capsule and the periaxial lymphatic space are formed throughout the whole length of the spindle.

In the denervated musculature of two rabbits, which had survived the operation, on the other hand, it is not possible to find the least trace of the differentiation of spindles after birth, i.e. 7 days after denervation.

The muscles are atrophic with small muscle-fibre diameters and without connective tissue proliferation (see Plate 2, fig. J). There are no structures nor end-to-end arrangements of nuclei in these muscles recalling the intrafusal fibres.

3. Section of *n. ischiadicus* in new-born rats

When the operation is performed in the new-born rat, the nuclear bag of the muscle-spindle is already differentiated.

Five days after neurotomy (4 animals) this differentiated equatorial segment is present, and only exceptionally can a reduction of the number of nuclei in some of the intrafusal fibres or symptoms of vacuolar degeneration be observed.

Ten days after neurotomy (3 animals) intrafusal fibres in the denervated muscles can hardly be discerned. The characteristic nuclear bag disappears completely and the intrafusal fibres disintegrate. In many cases an abundant invasion of phagocytic elements may be observed inside the spindle, which is formed by a connective tissue capsule closely adhering to the fragmenting intrafusal fibres.

4. Section of *n. ischiadicus* in 20-day-old rats

In the 20-day-old rats at the time of neurotomy the non-nervous component of the spindle is already morphologically mature.

Five days after the operation (4 animals) no changes were observed in the muscle-spindles of the denervated musculature.

Ten days after denervation (2 animals) slight atrophy of the muscle-fibres has occurred and a hardly noticeable shrinking of the nuclei in the equatorial zone.

DISCUSSION

It has been demonstrated that further differentiation of neuromuscular spindles is stopped by denervation performed in the initial stages of develop-

ment. In rare cases further division and arrangement of nuclei end-to-end in chains may take place in denervated myotubes, but the typical nuclear bag of the intrafusal fibre was never seen.

In new-born rats, in the musculature of which the nuclear bag of the intrafusal fibre is already almost differentiated, the intrafusal fibres atrophy in 10 days time and disintegrate. Innervation is evidently necessary for the normal course of development of the muscle-spindle.

In 20-day-old rats, on the other hand, the spindles of which are morphologically mature, it is possible to observe a hardly noticeable atrophy of the nuclear bag after 10 days. The loss of innervation leads then, at an early stage of development of neuromuscular spindles, to the arrest of the differentiation of intrafusal fibres. In new-born rats rapid disintegration of neuromuscular spindles occurs and in young rats gradual atrophy takes place as in adult animals.

In peripheral structures different degrees of trophic and morphogenetic dependence on innervation can be found. Non-nervous components of encapsulated skin receptors in mammals disintegrate after longer periods of denervation and their differentiation is again realized by the morphogenetic influence of regenerating nerve-fibres on the surrounding connective tissue in an even greater number than normal (Holobut & Jalowy, 1953; Dijkstra, 1933). According to these reports and our own experimental results, it seems probable that the trophic and morphogenetic dependence of receptors on their innervation may be greater than that of other peripheral structures.

The muscle-spindle as a receptor differs considerably from other encapsulated sensory endings in its structure as well as its innervation. The main difference lies in the fact that muscle-spindles have a dual innervation: proprioceptive in the equatorial zone and motor in the polar zones. By severing *n. ischiadicus* both the afferent and efferent innervation is affected. Is it possible under these experimental conditions to ascertain which of these two sets of nerve-fibres induces the differentiation of the spindles? This question may be answered by taking into account the normal development of muscle-spindle innervation. Motor innervation of intrafusal fibres is not completed until after birth, as has been demonstrated by impregnated sections and by the histochemical reaction to specific cholinesterase, which does not appear in the polar segments of muscle-spindles before the 5th day after birth (Zelená, 1957). Before birth the developing intrafusal fibres are only innervated by proprioceptive fibres with characteristic branching. The differentiation of muscle-spindles is stopped after intra-uterine denervation in consequence of the loss of proprioceptive nerve-fibres.

Is it possible that the retardation of muscle-spindle development is caused by loss of function, i.e. the specific function of the muscle, which cannot be realized after denervation? Our experiments do not answer this question directly, because section of *n. ischiadicus* removes motor as well as proprioceptive innervation of the muscle, so that only passive movements of the denervated extremity may be performed.

It has been shown, however, in denervation experiments on muscle-spindles of adult animals (Tower, 1932) that the elimination of proprioceptive nerve-fibres by extirpation of spinal ganglia leads to atrophy of the equatorial zone of the intrafusal fibres with a decrease in the number of nuclei even though motor innervation is intact. This atrophy therefore takes place, although specific function of the polar zone is preserved and the equatorial segment is tensed and relaxed as in normal spindles. If specific function does not prevent the atrophy of the equatorial segment in adult animals, it is possible to assume that neither during ontogenesis do nerve-fibres influence the differentiation of myotubes by way of the specific function, i.e. contraction of intrafusal fibres, since under normal conditions the polar zones of the spindle are not innervated until the differentiation of the equatorial segment had been roughly completed.

How then is the morphogenetic influence of the nervous system on the peripheral structures realized? The trophic influence of the nervous system is doubtless of a biochemical nature and is realized, most probably, as an effect of the nerve-endings on the metabolism of the innervated structure. The analysis of this influence is a difficult biochemical and histochemical problem. Analysis of the trophic influence of the motor neuron on skeletal muscle (Vodička, 1956) contributes, amongst others, towards a solution of the basic problem, the definition of the biochemical nature of the trophic influence of the nervous system.

It is interesting to note the different response of intrafusal fibres to denervation during differentiation and of intrafusal fibres in spindles morphologically mature. If the trophic influence of the nervous system is considered primarily to be in the activation of anabolic processes in tissue metabolism (Gutmann *et al.*, 1956), it is probable that during development and structural differentiation, where anabolic processes far surpass catabolic processes, the developing tissue is more sensitive to denervation because the activation of anabolic processes by the nervous system is eliminated. Adult structure is in comparison more in equilibrium as far as anabolic and catabolic processes are concerned, maintenance of the metabolic equilibrium is more stabilized, and denervation is followed by atrophy, i.e. by a gradual predominance of catabolic over anabolic processes.

SUMMARY

1. The development of muscle-spindles in the calf-muscles of the rat was followed under normal conditions and after section of n. ischiadicus.

2. The first differentiation of muscle-spindles in the muscles of the hind limb of the rat was observed on the 19th day of intra-uterine development and their morphogenesis is complete by the 25th day after birth.

3. After section of n. ischiadicus in rat foetuses on the 19th–20th day of intra-uterine development, differentiation of muscle-spindles does not take place; neither intrafusal fibres nor the connective tissue capsule are formed. Since only proprioceptive fibres have innervated the normal muscle at this time, it is the

loss of the morphogenetic influence of these nerve-fibres, that prevents differentiation. Similar results were obtained in rabbits denervated 7 days before birth.

4. When section of n. ischiadicus is performed in new-born rats, disintegration of intrafusal fibres in which the equatorial segment was already differentiated at the time of denervation occurs within 10 days.

5. Denervation in a 20-day-old rat, the muscle-spindles of which are already morphologically mature, leads after the same interval, to a slight atrophy in the equatorial zone of the intrafusal fibres.

6. The mechanism of the morphogenetic influence of the nervous system and its changes during ontogenesis are discussed.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Longitudinal section through the calf musculature of a rat 5 days old. *a*, spindle equatorial zone. Azan. $\times 200$.

FIG. B. Diagonal section through the neuromuscular spindle of a 10-day-old rat. *a*, equatorial zone; *m*, myoblastic zone; *c*, capsule. Haematoxylin-eosin. $\times 200$.

FIG. C. Longitudinal section of neuromuscular spindle in a 25-day-old rat. *a*, equatorial zone; *c*, capsule; I, primary endings at the equator; II, secondary endings in the myoblastic zone. Gross-Bielschowski. $\times 200$.

PLATE 2

FIG. D. Myotubes in the calf musculature of a rat foetus 3 days before birth. Van Gieson. $\times 1,100$.

FIG. E. The equatorial zone of an intrafusal fibre in a new-born rat. *c*, connective tissue capsule. Haematoxylin-eosin. $\times 1,100$.

FIG. F. Nuclei closely ranged end-to-end in myotubes of denervated musculature in a new-born rat 3 days after section of the sciatic nerve. Haematoxylin-eosin. $\times 1,100$.

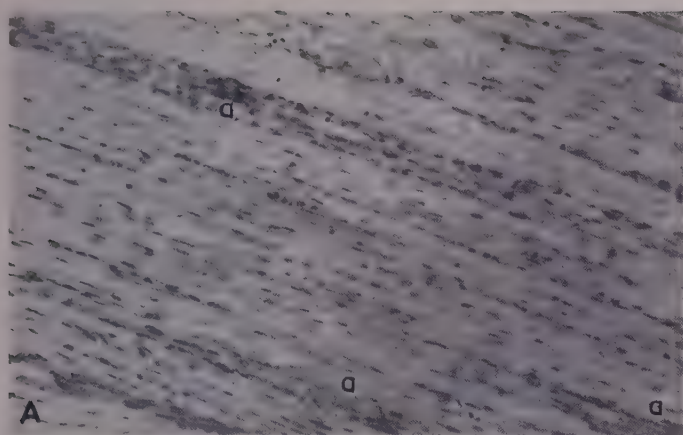
FIG. G. Chains of deformed nuclei in a swollen myotube of denervated musculature in a new-born rat 3 days after section of n. ischiadicus. Haematoxylin-eosin. $\times 1,100$.

FIG. H. Myotubes in a rabbit foetus 7 days before birth. *a*, chains of nuclei in myotubes. Haematoxylin-eosin. $\times 400$.

FIG. I. The central segment of a spindle in a new-born rabbit. *a*, equatorial zone; *m*, myotubal zone; *c*, capsule. Haematoxylin-eosin. $\times 400$.

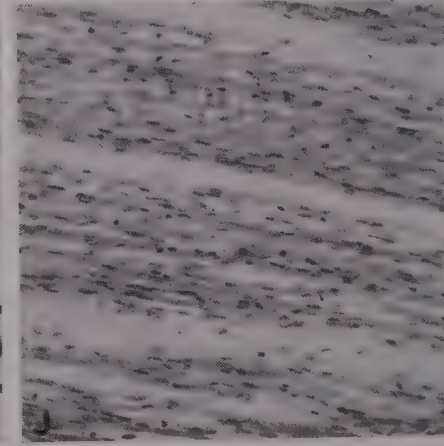
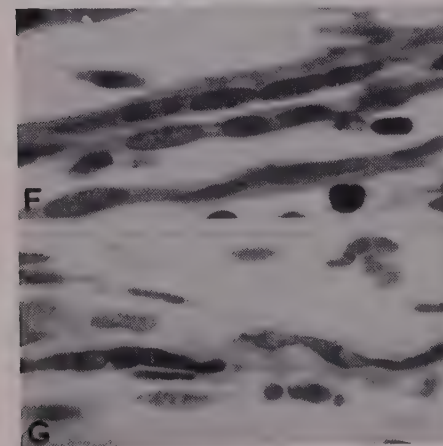
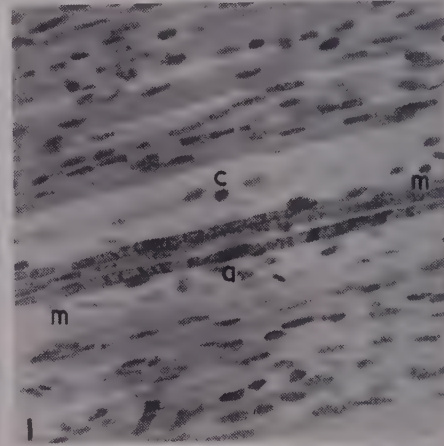
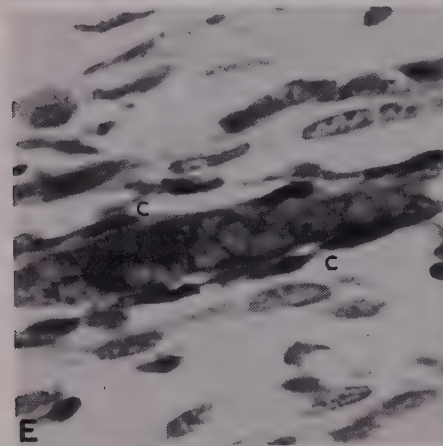
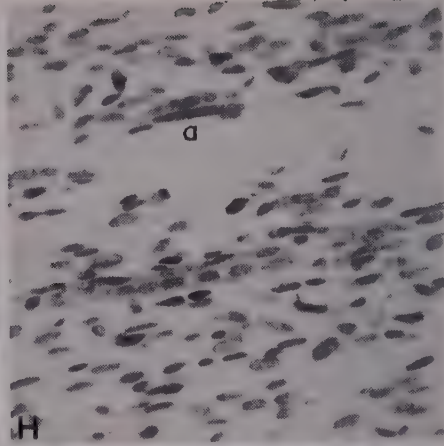
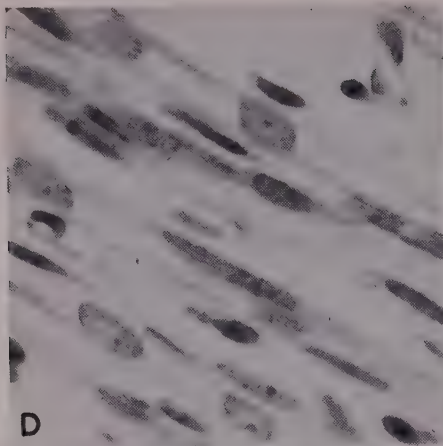
FIG. J. The atrophy of denervated musculature in a new-born rabbit 7 days after section of the sciatic nerve. Haematoxylin-eosin. $\times 200$.

(Manuscript received 23 : x : 56)



J. ZELENÁ

Plate 1



J. ZELENÁ
Plate 2

A Critical Period for the Turning of Hens' Eggs

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INTRODUCTION

THE artificial incubation of hens' eggs involves four factors—temperature, humidity, air supply, and at intervals a rotation or 'turning' of the eggs. This last factor is perhaps the most curious and unexpected of the four, yet there is no doubt that it is necessary for development as shown by good hatchability, and in the natural state it is carried out by the sitting hen. Eycleshymer (1906), Chattock (1925), and Olsen (1930) have all concluded from observations on the hen's nest that the hen frequently rotates the eggs during the incubation period; Olsen considers it occurs as often as 96 times in 24 hours.

Various abnormalities have been recorded in eggs incubated without turning. Dareste (1891) stated that absence of turning causes the allantois to adhere to the yolk sac; Eycleshymer (1906) confirmed this, and added that during the first week of incubation, absence of turning may also cause the embryo to adhere to the shell membranes. Randles & Romanoff (1950) have observed further effects: in unturned eggs the formation of the albumen sac is delayed and the physical properties of the amniotic and allantoic fluids differ from normal.

Although the relation between frequency of turning and hatchability has been intensively studied (see Landauer, 1951, pp. 54–55 for references), little appears to be known as to the mechanism by which insufficient turning induces abnormalities. A useful line of approach to this question would be to find the relative importance of turning eggs at different stages of the incubation period. If it were known that at a particular stage of development the embryo was exceptionally sensitive to the absence of turning, it might be possible to deduce what the function of turning was from a consideration of the condition of the egg contents at that time; or at least the range of possible explanations might be greatly narrowed. Such few indications as have been obtained previously suggest that turning is more important in early than in late incubation. For example, Byerly & Olsen (1936) conclude that turning in the third week probably has little effect on hatchability, and Card (1926) observed that eggs turned during the first 6 days hatch nearly as well as those turned throughout incubation, but he gives no figures.

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Consideration of the arrangement of the egg contents suggests that a particularly critical time for turning the eggs might be the latter part of the first week of incubation. At this time a large area of chorion lies close to the shell membranes and the layer of albumen between the two has been greatly reduced by a loss of fluid from the albumen to the yolk. Abnormal adhesions between the chorion and shell membranes, therefore, seem a possibility at this stage unless the shell and its membranes are periodically moved relative to the egg contents, i.e. unless the eggs are turned. The present work consists of evidence in support of this explanation. It will be described in two sections, the first indicating that the most important time for turning the eggs is between the 4th and 7th days of incubation, and the second suggesting that turning during this period exerts its marked effect on hatchability by preventing harmful adhesions arising between the chorion and shell membranes.

METHOD AND RESULTS

(a) *Hatchability of eggs turned only between the 4th and 7th day*

Table 1 summarizes the results of four experiments designed to test the effect on hatchability of turning the eggs only between the 4th and 7th day (inclusive). The eggs were incubated at 39° C. (heated from above), at about 60 per cent. relative humidity, and turned on the days indicated. Turning was carried out once every 12 hours and the eggs rotated by hand approximately 120° about the long axis; they were rotated alternately right and left.

TABLE 1

Hatchability of eggs after various turning treatments during incubation

Experiment No.	Turned 4th–7th day		Turned 8th–11th day		Turned throughout		Unturned	
	Failed	Hatched	Failed	Hatched	Failed	Hatched	Failed	Hatched
1 . .	2	6	5	3	2	6	4	4
2 . .	2	7	7	2	5	4	8	1
3 . .	6	3	9	0	2	7	6	3
4 . .	4	5	8	1	2	7	8	1
TOTALS .	14	21	29	6	11	24	26	9

In each experiment the eggs were divided into four equal groups. The first group was turned between the 4th and 7th day, the second between the 8th and 11th day, the third throughout incubation up to the 18th day, and the fourth was not turned at all. The eggs of the four groups were interspersed to avoid differences arising from microclimates within the incubator. The purpose of including a group turned only between the 8th and 11th day was to find whether any improvement in hatchability of the 4–7 day group was restricted to eggs turned specifically during this period, or whether it resulted from turning them

during any 3-day period at about the middle of incubation. Turning of the 'turned-throughout' group was stopped at the 18th day to conform with the normal practice in poultry rearing.

The results of the four experiments added together show clearly that turning the eggs between the 4th and 7th day is similar in its effects on hatchability to turning them throughout the whole incubation period, and turning the eggs only between the 8th and 11th day gives similar results to leaving them completely unturned. Calculation of χ squared values for these two pairs of results also fails to detect significant differences. But χ squared calculated for a comparison of the results of turning 4th to 7th day with those of turning 8th–11th gives a P value of less than 0.001. It seems unquestionable, therefore, that turning the eggs between the 4th and 7th day of incubation gives an improvement in hatchability similar to that obtained by turning the eggs throughout incubation, and the results further suggest that this improvement is limited to eggs which are turned specifically at this stage.

In adding the results of separate experiments together the question of homogeneity arises. The numbers involved in each experiment are here too small to test for homogeneity by the χ squared method. However, if for each treatment the two results are selected which show the greatest difference, it can be shown that the probability of their belonging to different populations is insignificant; e.g. the extreme cases resulting from turning between the 4th and 7th day were 2 failed, 7 hatched; and 6 failed, 3 hatched. The probability of these belonging to the same population is 0.0767.

TABLE 2
Distribution of mortality during incubation

	<i>Infertile</i>	<i>Died 1st week</i>	<i>Died 2nd week</i>	<i>Died 3rd week</i>	<i>Hatched</i>
Turned 4th–7th day .	4	1	2	7	21
Turned 8th–11th day .	7	7	7	8	6
Turned throughout .	4	5	0	2	24
Unturned . . .	6	3	2	15	9

The eggs that did not hatch were opened on the 25th day and the time of death estimated from the extent of embryonic development. Table 2 shows the totals from the four experiments. The division between 'infertile' and 'died 1st week' can only be considered as roughly established since in those cases where the blastoderm died very young it is not always possible to distinguish the egg contents from the infertile condition, particularly after 3 weeks of incubation. The table shows that by far the heaviest mortality of unturned eggs occurs during the third week and it is this mortality which is most affected by turning throughout incubation or from the 4th to 7th day. The results also suggest that turning from the 8th to 11th day reduces the third-week mortality but correspondingly in-

creases mortality in the second week; however, the figures are not large enough to constitute a convincing proof of this.

In those eggs which had died after the 18th day of incubation, malpositions of the chick were fairly common. Of the 26 dead chicks in this group, 6 were found to have the head in the small end of the egg, 4 to have the beak away from the air sac, 3 to have the head under the left wing, and 1 to have the head between the thighs.

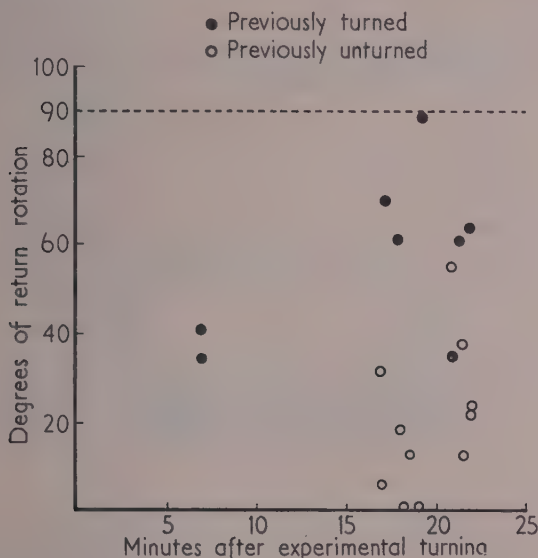
(b) Adhesion between the chorion and shell membranes

Statements that insufficient turning leads to adhesions between the chorion (or blastoderm) and shell membranes have frequently been made (e.g. Eycleshymer, 1906) but it is not quite clear what evidence they are based on. The chorion is very close to the shell membranes whether the eggs are turned or unturned, and in the second week of incubation the chorio-allantois adheres to the shell membranes as a normal feature of development. Furthermore, a small proportion of unturned eggs develop successfully and produce normal chicks at hatching. It would need, therefore, fairly carefully controlled observations to be certain that abnormal adhesions were occurring.

With this in mind the following experiment was devised. Two groups of fertile eggs were incubated for 7 days. The first group (11 eggs) was left unturned during this period; the second group (8 eggs) was turned every 12 hours between the 4th and 7th day (inclusive). On the 7th day all the eggs were candled and the position of the embryonic membranes marked by drawing a pencil line on the shell over some prominent blood-vessel (usually the sinus terminalis). Each egg was then rotated through 90° and left in this position for a few minutes. On candling for the second time the embryo was seen to have begun to move back towards its former position at the top of the egg, and the embryonic membranes had correspondingly moved relative to the pencil mark on the shell. By measuring with calipers the distance moved by the membranes and also the circumference of the egg, sufficient data were provided for calculating the angle through which the egg contents had turned. The results are plotted in the accompanying graph (Text-fig. 1), which shows the angle through which the egg contents have rotated towards their former position at a given time after the egg was turned through 90° . It can clearly be seen that in the eggs that were unturned during the period of incubation, movement of the embryonic membranes relative to the shell is very much slower than in eggs that were turned between the 4th and 7th days; in fact two of the 'unturnd' group have not moved at all.

The eggs were returned to the incubator and examined again 3 hours later. In all those of the 'turned' group the contents had moved through more than 80° , whilst in over half those of the 'unturnd' group the contents had rotated less than 60° . The only explanation that seems reasonable is that turning the eggs between the 4th and 7th day of incubation lowers the resistance to relative

movement of the chorion and shell membrane; or in other words, absence of turning encourages adherence between the two.



TEXT-FIG. 1. Each egg was turned through 90° . The subsequent internal rotation by which the egg contents returned towards their former position is plotted (in degrees) against time after the whole egg was turned. Of the two groups of eggs shown, one had been systematically turned during incubation, the other had not.

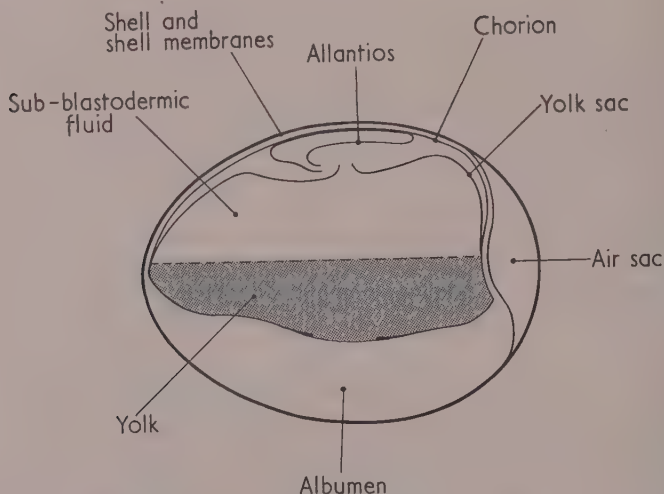
After these observations had been made, incubation of the eggs was continued without any further turning. Of the unturned group (actually turned once through 90° during the experiment on the 7th day) 2 out of 11 eventually hatched. Of the group turned between the 4th and 7th day 6 out of 8 hatched. This lends further support to conclusions reached in the previous section.

DISCUSSION

It is clear that the turning of hens' eggs is particularly important during the latter half of the first week of incubation. If the eggs are turned at this time good hatchability is obtained even if they are left unturned during the remainder of the incubation period. If they are not turned at all during incubation, however, hatchability is low. It has been shown that absence of turning results in an abnormal degree of adhesion between the chorion and shell membranes during the first week of incubation; the probable inference is, therefore, that this abnormal adhesion is closely connected with the causes of the high mortality found in unturned eggs.

Yet there remains the curious fact that, during the second week of incubation,

firm adhesion between the chorion (chorio-allantois) and the shell membranes occurs as a normal feature of development. This normal adhesion is evidently primarily related to the respiratory needs of the embryo. It is important that respiratory surfaces should be applied as closely as possible to the shell; in fact it has been shown (Danchakoff, 1917) that the allantoic capillaries actually move to a position outside the chorionic ectoderm.



TEXT-FIG. 2. Distribution of some of the egg contents at the 6th day of incubation. Based on a sagittal section of an egg fixed by boiling. (For the sake of clarity the distance between the chorion and shell membrane has been exaggerated.)

It seems, therefore, that turning is not a device to prevent adherence altogether, but to prevent it occurring too soon. Why is this so important? The answer may be in the state of development of the chorion and yolk sac during the first and second weeks. Although these two membranes have practically surrounded the yolk by the 4th day, they continue to expand rapidly until the end of the 6th day (Grodzinski, 1934) owing to the increase of the yolk-sac contents as a result of sub-blastodermic fluid formation. Probably, therefore, any adhesion of the chorion to the shell membrane before the 6th day would hinder its normal development, but after this time would not be so serious.

Part of the mechanism involved in premature adhesion is likely to be the rapid water loss from the albumen which is occurring at this time. Until the end of the first week the albumen is constantly losing water (*a*) by evaporation from the shell surface, and (*b*) via the blastoderm to form the sub-blastodermic fluid (New, 1956). Since the albumen layer between much of the chorion and shell membranes has become extremely thin by the end of the first week of incubation and the area of chorion close to the shell membrane has become very large (Text-fig. 2), desiccation of this albumen layer would seem greatly to increase the

chances of adhesion. Turning the egg at this stage might well prevent such desiccation by supplying a fresh layer of watery albumen over the dry regions of the membranes.

As mentioned previously a variety of abnormalities have been observed in unturned eggs. The present work suggests, however, that many of these abnormalities are likely to be secondary effects of an initial interference with development occurring between the 4th and 7th day.

SUMMARY

1. Turning hens' eggs between the 4th and 7th day of incubation gives a hatchability similar to that of eggs turned throughout incubation.

2. Turning the eggs between the 8th and 11th day gives a hatchability similar to that of unturned eggs.

3. New evidence is presented that absence of turning leads to abnormal adhesion between the chorion and shell membranes.

4. The mechanism by which turning effects development is discussed.

ACKNOWLEDGEMENTS

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Contribution à l'étude de la maturation et de la segmentation de l'œuf d'Aplysie

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INTRODUCTION

LA segmentation rigoureusement déterminée des Spiralia est caractérisée par une rotation alternante, spirale des micromères au moment de leur émission. De plus, on constate des divisions inégales caractéristiques pour chaque espèce et qui ne sont pas une simple conséquence de la condensation de vitellus en certains endroits de l'œuf. En ce qui concerne plus en particulier la première division de segmentation, celle-ci peut être égale ou inégale, selon le genre envisagé, chez les Polychètes et les Gastéropodes. Elle est toujours égale chez les Némertiens, toujours inégale chez les Hirudinées, les Oligochètes, les Siponculiens, les Géphiriens, les Rotifères et les Lamellibranches. Lorsqu'elle est inégale, cette première division favorise le plus souvent le blastomère CD, celui dont naîtra à la seconde division le blastomère D, à partir duquel se développera la lignée mésoblastique. Cependant chez les Gastéropodes opisthobranches à première division inégale, celle-ci fournit un blastomère AB, qui quoique purement ectoblastique et entoblastique, est plus volumineux que le blastomère CD. C'est notamment le cas d'Aplysia.

Ce caractère égal ou inégal de la première division de segmentation est indubitablement lié à la morphogénèse. Dans des expériences déjà anciennes, Penners (1924) et Tyler (1930) ont montré que l'égalisation de cette division, chez des genres où elle est normalement inégale, provoque la formation de monstres doubles. Schleip (1929) tend à mettre la segmentation inégale en rapport avec des 'plasmes polaires', qui ont une valeur de localisation germinale. Deux plasmes polaires bien différenciés et localisés, l'un au pôle animal, l'autre au pôle végétatif, ont été signalés par l'école de Schleip chez les Hirudinées et les Oligochètes. Ils passent dans le blastomère CD, ensuite dans le quadrant D, pour se localiser enfin dans les cellules-souches des bandes germinatives. Lorsque dans d'autres groupes un plasma polaire existe, il est végétatif, et s'accompagne de la formation d'un lobe polaire au moment des premières divisions de segmentation. Ce plasma polaire passe également dans CD et puis dans D.

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Pasteels (1934) a étudié expérimentalement le mécanisme de la segmentation inégale quelque peu particulier d'Aplysie. Il arrive à admettre comme hypothèse logique chez *Aplysia*, une répartition et une condensation différentes de substances analogues de celle des plasmes polaires, capables d'opposer une certaine résistance aux processus cytodierétiques et ayant, comme les plasmes polaires, la valeur de localisations germinales. L'explication du lien entre la segmentation inégale et la morphogénèse serait donc, ici aussi, assurée de ce fait.

Les observations qui vont suivre nous ont incité à reprendre cette question.

OBSERVATIONS

Les observations que nous relatons ici ont été effectuées à la Station biologique de Roscoff entre le 19. v et le 31 v. 1956. Après un hiver exceptionnellement rigoureux, la température du laboratoire oscillait entre 15° et 18° C. Dans ces conditions il nous a été possible de recueillir et de suivre de nombreuses pontes d'*Aplysia punctata*. La maturation et les premiers stades de la segmentation évoluent très lentement. Sur des filaments prélevés au moment de la ponte on constate l'expulsion du premier globule polaire après environ 4 heures, celle du second globule polaire après environ 6 heures. Après à peu près 8 heures surviennent les déformations qui annoncent la première division de segmentation. Après 24 heures on observe les stades à 3 blastomères, suivis plus ou moins rapidement de ceux à 4 et à 6 blastomères.

Les divers stades de la maturation et les premiers stades de la segmentation furent étudiés à l'aide de colorations vitales. Nous avons utilisé le Vert Janus, le Rouge neutre, le Bleu de Toluidine à une concentration de 1/15 000 dans de l'eau de mer, au pH de cette dernière (pH 7,8). En dilacérant les filaments, on libère les œufs à divers moments de leur développement dans les solutions colorées, où ils restent de 15 à 20 minutes avant d'être examinés au microscope. Les deux premiers colorants vitaux ne nous ont montré rien de bien particulier. Le Vert Janus colore les mitochondries, qui sont très abondantes dans l'exoplasme; on les voit cependant tout aussi nettement à l'examen direct au microscope sans aucun traitement préalable. Le Rouge neutre colore tout l'œuf de manière diffuse. On a parfois l'impression que le colorant se concentre quelque peu dans des vacuoles accolées aux mitochondries de l'exoplasme. Dans un seul cas, nous avons pu observer un œuf au stade de 3 blastomères, où l'exoplasme des blastomères C et D était resté incolore pour des raisons qui nous échappent. Dans ce cas des 'vacuoles à rouge neutre' accolées aux mitochondries tranchaient très nettement sur le fond incolore.

Plus instructives furent les colorations au bleu de Toluidine; ce sont elles qui formeront plus en particulier le sujet de ce travail. On sait que ce colorant peut teinter *in vivo* certains éléments de manière orthochrome (en bleu), d'autres de manière métachromatique (en pourpre). Bien qu'il soit possible en analysant les conditions de pH et de concentration, dans lesquelles elle survient, de préciser quelque peu la signification histochimique de la réaction métachromatique *in*

vivo (Fautrez-Firlefyn, 1954), il est dangereux de lui donner sans plus une valeur précise. Aussi nous sommes nous contenté dans ces observations de mettre en évidence certaines différenciations dans le cytoplasme de l'œuf d'Aplysie, sans en rien vouloir préjuger de leur nature.

L'œuf fraîchement pondu est très régulièrement sphérique. On y distingue un endoplasme opaque et légèrement brunâtre, entouré d'une mince couche d'exoplasme hyalin. Après coloration au bleu de Toluidine, l'endoplasme se teinte de manière homogène en pourpre, ce qui lui confère une coloration sale, la teinte métachromatique du bleu se superposant à la teinte de fond naturelle brunâtre. L'exoplasme au contraire prend une très belle coloration pourpre transparente. On a l'impression que cet exoplasme est plus avide du colorant que l'endoplasme. Il est cependant très difficile de se faire une opinion très franche à ce propos, la teinte brunâtre et le caractère opaque de l'endoplasme faussant assez fortement l'observation. Notons dès à présent, pour ne plus y revenir, que pendant tous les stades ultérieurs que nous avons étudiés, l'endoplasme des œufs colorés au bleu de Toluidine garde le même aspect, et que son caractère opaque rend toute observation détaillée impossible à son niveau par la technique utilisée. Celle-ci ne peut donc nous fournir des renseignements que sur les rapports globaux entre endoplasme et exoplasme, et sur des processus qui se déroulent dans l'exoplasme transparent.

Immédiatement après la ponte, l'œuf est couvert de toutes parts d'une mince couche bleu-ciel après coloration vitale. Cette couche comprend le film limitant et une bande très mince de cytoplasme fondamental. L'ensemble n'apparaît à premier abord que comme un reflet bleuâtre à la surface de la partie pourpre de l'exoplasme, infiniment plus épaisse. Cet exoplasme est bourré de mitochondries en filaments assez longs et sinueux, au contact desquelles se forment de minuscules vacuoles. Ces mitochondries se colorent en pourpre comme le plasmе fondamental dans lequel elles baignent. Elles ne pénètrent pas dans la bande superficielle du plasmе orthochrome.

Très rapidement les conditions changent. Le reflet bleuâtre disparaît, et l'exoplasme, qui gonfle légèrement, devient métachromatique sur toute son épaisseur. Cet aspect (Fig. 1a) persiste jusqu'au moment où surviennent les phénomènes qui vont préluder à l'expulsion du premier globule polaire.

Ces phénomènes amènent une série de profondes modifications schématisées dans la Fig. 1b. A un pôle de l'œuf, l'endoplasme présente une excavation assez prononcée. Initialement la surface de l'œuf n'est pas déformée à ce niveau et l'accumulation d'exoplasme n'y est que le fait de l'excavation endoplasmique. Plus tard cependant la surface ovulaire va bomber légèrement vers le dehors et l'exoplasme acquiert ainsi l'aspect d'une lentille biconvexe. Après coloration au bleu de Toluidine, ce plasmе lenticulaire se différencie très nettement du reste de l'œuf par le fait qu'il reste absolument incolore. Il apparaît comme un plasmе hyalin, incolore et dans lequel baignent de nombreuses mitochondries, au contact desquelles on n'observe que de rares et minuscules vacuoles. L'ensemble de

l'exoplasme présente des modifications qui s'extériorisent après coloration vitale par la réapparition de la bande superficielle orthochrome, constituée du film limitant et d'une très mince couche de plasmé sous-jacent. Cette bande bleue ne couvre pas le plasmé incolore. Sa situation est schématisée par la partie épaisse du contour de l'œuf. (Cette représentation n'a bien entendu qu'une valeur schématique; le trait du dessin est infiniment plus large que l'épaisseur relative

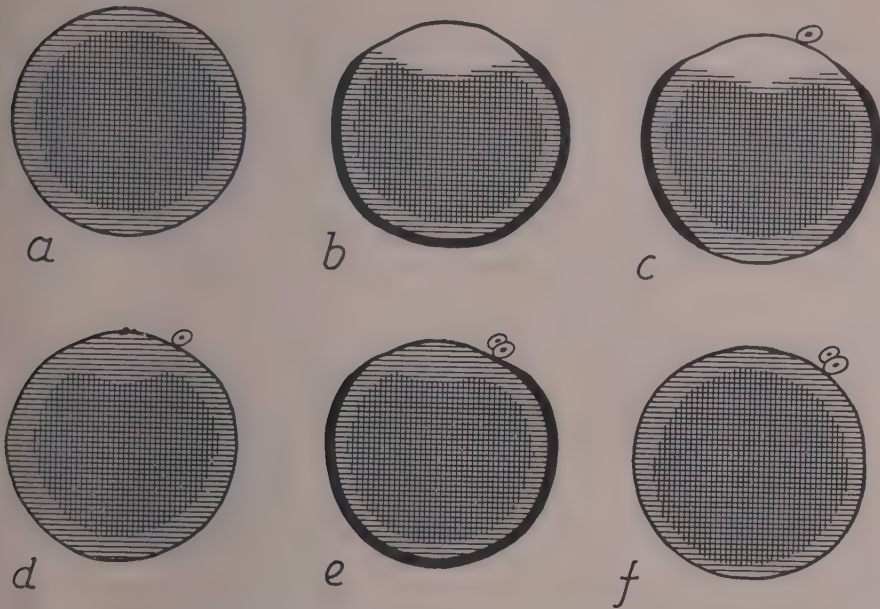


FIG. 1. La maturation de l'œuf d'*Aplysia punctata* (schémas d'après des dessins à main levée au microscope). Quadrillé: endoplasme. Strié: exoplasme pourpre. En blanc: exoplasme incolore. Traits épaissis: zone orthochrome cortico-sous-corticale (le trait schématique est beaucoup plus épais que la couche dont il indique la situation).

de la couche orthochrome.) La plus grande partie profonde de l'exoplasme reste métachromatique. Elle contient des mitochondries et d'assez volumineuses vacuoles, accolées à celles-ci. Notons que cette couche métachromatique contourne complètement l'endoplasme en passant entre ce dernier et le plasmé incolore.

Le premier globule polaire est expulsé dans la région du plasmé hyalin (Fig. 1c). Notons cependant que le pôle de maturation n'est jamais localisé au centre de ce plasmé, mais se situe au contraire très près de son bord. Le cytoplasme du globule polaire prend la teinte métachromatique. Son noyau est initialement bleu; le colorant y souligne très nettement le réticulum de chromatine. Très rapidement ce noyau se condense en une masse pycnotique et virée au pourpre. Assez fréquemment cependant ce premier globule polaire se divise en deux.

Après l'expulsion du premier globule polaire, on assiste à de nouvelles modifications de l'exoplasme. La couche orthochrome disparaît, et ceci à partir de l'antipole. La large calotte est remplacée par un anneau, qui se rétrécit rapidement en s'éloignant de l'antipole. Toute l'épaisseur de l'exoplasme redevient pourpre; il en est de même de l'accumulation exoplasmique près du pôle de maturation (Fig. 1*d*).

Au moment de l'expulsion du deuxième globule polaire (Fig. 1*e*) on peut observer dans l'exoplasme des phénomènes assez semblables à ceux qui accompagnent l'expulsion du premier globule polaire; ils sont toutefois plus discrets. Signalons une nouvelle et dernière réapparition de la bande corticale orthochrome. Le cône d'expulsion est moins net que lors de la formation du premier globule polaire; il reste métachromatique. Au point de vue de sa coloration le second globule polaire est absolument semblable au premier.

Après son expulsion (Fig. 1*f*), tout l'exoplasme redevient encore une fois complètement métachromatique. L'excavation endoplasmique s'estompe et l'aspect général de l'œuf redevient semblable à ce qu'il était avant les divisions de maturation. On a seulement l'impression dans la plupart des œufs que la couche exoplasmique est plus mince à l'antipole et que graduellement elle s'épaissit pour atteindre un maximum de largeur au pôle de maturation, porteur des globules polaires.

Environ deux heures plus tard survient une déformation spectaculaire de l'œuf. On voit apparaître un véritable lobe exoplasmique. De sphérique, l'œuf devient plutôt ovoïde, avec un étranglement large et peu profond sous son extrémité étroite, exoplasmique (Fig. 2*a*). C'est dans cet étranglement que l'on retrouve constamment les globules polaires. La condensation exoplasmique est donc nettement déviée par rapport au pôle de maturation. Comme on le verra plus loin, il va constituer une grande partie du petit blastomère CD. La coloration vitale au bleu de Toluidine montre que ce lobe est constitué par un véritable plasme, qui se distingue du reste de l'exoplasme par le fait qu'il ne prend pas le colorant. Incolore et hyalin, il tranche nettement sur l'exoplasme qui entoure de toutes parts l'endoplasme.

Bientôt l'œuf se déforme encore. Vis-à-vis du lobe hyalin, de l'autre côté des globules polaires la surface se bombe par extension du plasme incolore, de telle sorte que l'œuf devient pratiquement symétrique par rapport à un plan, qui passe par les globules polaires (Fig. 2*b*). La disposition des plasmes n'est cependant pas symétrique par rapport à ce plan. Du côté de l'ancien lobe hyalin, reste accumulée la plus grande partie du plasme incolore. Ce dernier cependant tend à envahir une partie de l'œuf située de l'autre côté des globules polaires. Le sillon de la première cytotidérèse progresse entre-temps. Parfois large et profond, il réalise le stade en U ou même en fer à cheval. Il finit par s'incliner sous l'ancien lobe hyalin, et ainsi est réalisé le stade à deux blastomères: grand blastomère AB et petit blastomère CD (Fig. 2*c*). Dans ces deux blastomères le rapport entre les divers plasmes est ainsi dès le début très différent. Le blastomère AB est essen-

tiellement composé d'endoplasme, dont il emporte la plus grande partie. Cet endoplasme est entouré d'une couche assez régulière d'exoplasme pourpre, riche en mitochondries auxquelles sont accolées de grosses vacuoles. Près du pôle animal il ne contient qu'une assez petite calotte d'exoplasme incolore. Le blastomère CD par contre contient surtout du plasme incolore; la moitié végétative seule est composée d'endoplasme, entouré d'exoplasme pourpre.

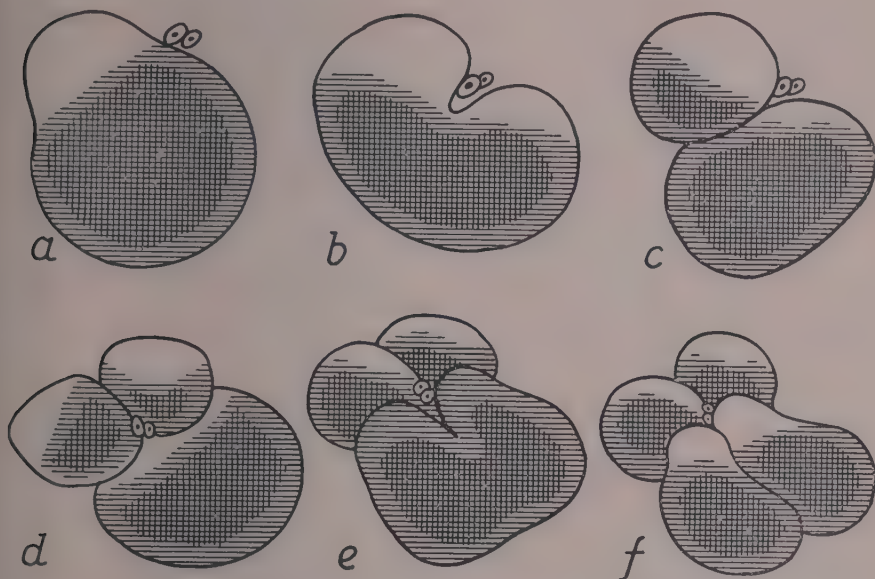


FIG. 2. Début de la segmentation de l'œuf d'*Aplysia punctata*. Quadrillé: endoplasme. Strié: exoplasme pourpre. En blanc: exoplasme incolore.

Dans toutes nos observations nous avons pu noter un asynchronisme entre la division du blastomère CD et celle du blastomère AB. La division plus précoce du blastomère CD réalise un stade à trois blastomères (Fig. 2d). La cytodierèse s'aperçoit d'abord par une encoche à la partie équatoriale du blastomère CD et qui se rapproche rapidement du premier sillon de segmentation. A ce moment la limite entre endoplasme et exoplasme est moins nette. A la fin de la division elle redevient plus tranchée par tassement des éléments opaques profonds. La répartition des divers plasmes est encore une fois inégale entre les deux blastomères C et D. Ils contiennent tous deux vers le pôle animal de l'exoplasme incolore. Celui-ci est beaucoup plus abondant dans le blastomère D que dans le blastomère C. L'endoplasme, toujours entouré d'exoplasme pourpre, dévié vers le pôle végétatif (et représenté, vu par transparence à travers du plasme incolore sur le schéma 2d), présente une répartition inverse entre les deux cellules.

Ce n'est que plus tard que s'amorce la cytodierèse au niveau de AB (Fig. 2e).

A ce moment le plasma incolore y a complètement disparu et l'endoplasme n'y est entouré que de l'exoplasme pourpre, riche en mitochondries et vacuoles. Les deux plasmas semblent se répartir de manière égale entre les blastomères A et B. Dans les blastomères C et D les rapports entre les plasmas restent inchangés.

A un stade ultérieur, on observe une déformation de chacun des blastomères A et B dirigée comme un petit lobe vers le pôle animal (Fig. 2f). Cette déformation n'est pas sans rappeler celle qui précède, dans l'œuf, la première division de segmentation. Elle la rappelle d'autant plus, qu'après coloration vitale on constate que les lobes animaux sont encore une fois formés d'exoplasme qui reste absolument incolore, tandis que la grande masse de chaque blastomère est de l'endoplasme, entouré d'exoplasme pourpre. L'exoplasme incolore passe presque complètement dans les micromères 1a et 1b, dont il forme la plus grande partie. Remarquons, que nous avons toujours vu cette formation de micromères 1a et 1b précéder celles des micromères 1c et 1d.

A des stades plus avancés de la segmentation, les micromères sont transparents, formés d'un exoplasme pourpre rempli de minces mitochondries, tandis que la masse des macromères est opaque, formée d'un endoplasme entouré d'exoplasme pourpre, dans lequel les mitochondries sont plus massives et accolées à de nombreuses vacuoles.

DISCUSSION

Le cell-lineage a été nettement établi chez *Aplysia* par Carazzi (1905). Nous n'aurons qu'à signaler quelques différences dans la chronologie des premières divisions de segmentation entre nos observations sur l'*Aplysia punctata* de Roscoff, et celles de cet auteur sur les trois espèces d'*Aplysie* à Naples. C'est ainsi que nous avons constamment trouvé un stade à trois blastomères, du fait que la division de CD précède de loin celle de AB. En outre nous avons toujours observé la formation des micromères 1a et 1b longtemps avant celle de 1c et 1d, contrairement à ce qu'indique Carazzi.

En ce qui concerne la constitution du cytoplasme ovulaire, la distinction entre un endoplasme dense et opaque et un exoplasme fluide et transparent ne semble pas avoir retenu l'attention des observateurs. Tout au plus Raven (1938) parle-t-il d'une mince couche superficielle de vitellus à grains fins dans l'œuf d'*Aplysia depilans*. La distinction entre les deux plasmas nous est cependant toujours apparue très nette à l'examen *in vivo*, où l'exoplasme transparent est riche en longues mitochondries et en vacuoles, tandis que l'endoplasme se présente comme une masse opaque.

Ce sont surtout les différenciations qui apparaissent dans cet exoplasme, et qui sont soulignées ou révélées par l'usage du bleu de Toluidine en coloration vitale, qui ont retenu notre attention. Dès le moment de la ponte et au cours de la maturation, un examen attentif permet de suivre des transformations exoplasmaïques, qui sont de toute évidence en rapport avec la constitution des cônes

d'expulsion des globules polaires. La plus nette de ces transformations est l'accumulation d'un plasma qui ne se colore pas au bleu de Toluidine (et que nous appellerons pour la facilité de l'exposé 'plasma incolore') près du pôle de maturation lors de l'expulsion du premier globule polaire. Sans vouloir préjuger de ce qui pourrait se passer à ce moment dans l'endoplasme, et qui échapperait à notre investigation, on ne peut qu'admettre que l'exoplasme entier joue un rôle dans l'élaboration de ce plasma incolore. Cette élaboration s'accompagne en effet d'un retassement vers la profondeur de mitochondries et vacuoles baignant dans un plasma métachromatique, avec dégagement d'une couche cortico-sous-corticale dépourvue d'inclusions et sur laquelle un reflet orthochrome attire l'attention. Cette diversification rétablit la situation qui semble exister dans l'œuf au moment de la ponte, et s'efface, après l'expulsion du globule polaire, assez nettement à partir de l'antipole. Au moment de l'expulsion du second globule polaire, les modifications sont moins prononcées, et le plasma du cône d'expulsion ne devient jamais aussi nettement incolore. Notons enfin que l'accumulation d'exoplasme n'est jamais strictement polaire, en ce sens que les globules polaires sont toujours expulsés près de sa périphérie. Un certain décalage existe donc entre polarité cytoplasmique et le pôle de maturation.

Mais c'est après la maturation qu'apparaissent les modifications les plus spectaculaires. Elles correspondent aux 'changements tant morphologiques que physiologiques' observés à ce moment par Pasteels (1934). Le dégagement de presque toute la moitié animale de vitellus, qui correspond sur l'œuf *in vivo* à l'apparition d'un pôle clair extrêmement caractéristique, et sur lequel cet auteur attire l'attention, s'observe avec une grande netteté après coloration vitale au bleu de Toluidine. C'est le stade très typique représenté sur la fig. 2a. Dès le début nous voulons cependant insister sur le fait que le pôle clair, envahi par un plasma incolore, qui fait bomber la surface de l'œuf, ne se trouve pas au futur pôle animal. Celui-ci va se situer à l'endroit du pôle de maturation, indiqué par la situation des globules polaires. Or ces globules polaires se retrouvent *toujours* dans le large sillon qui circonscrit la déformation due à l'accumulation du plasma clair. Le pôle clair est donc dévié par rapport du pôle animal, et dès maintenant il est possible de dire que c'est de son côté que se formera le blastomère CD. La succession des fig. 2 a, b et c schématise cette évolution. On y retrouve la localisation du plasma incolore, de l'exoplasme pourpre et de l'endoplasme. Après le stade à plasma incolore proéminent d'un côté du pôle animal (Fig. 2a), on assiste à une tendance à la symétrisation par rapport au pôle animal (Fig. 2b). Tandis que le premier sillon de segmentation s'approfondit, l'œuf en son ensemble devient en réalité symétrique par rapport à celui-ci, mais la répartition des plasmas reste oblique par rapport au plan de ce sillon. Le plasma incolore qui se forme du côté destiné à AB reste peu abondant. Lorsque enfin le sillon de segmentation s'incurve sous le futur blastomère CD (Fig. 2c), rendant ainsi la cytodierèse inégale en faveur de la cellule AB, on voit une nette différence dans la répartition des divers plasmas entre les deux premiers blastomères. *Le petit*

blastomère CD emporte la presque totalité du plasma incolore; il contient relativement peu d'endoplasme, entouré de toutes parts d'exoplasme pourpre. Le blastomère AB au contraire ne contient que fort peu de plasma incolore, et beaucoup d'endoplasme entouré d'exoplasme pourpre.

Jusqu'à ce moment on a l'impression qu'un plan de symétrie bilatérale pourrait exister dans ces germes: il coïnciderait avec le plan du papier dans les schémas 2 a, b, c. La division du blastomère CD attire cependant l'attention sur le fait que la séparation entre l'exoplasme pourpre et le plasma incolore est vraisemblablement aussi oblique dans un plan perpendiculaire, qui passant par le pôle animal suit le plan de clivage entre AB et CD. Cette séparation se trouverait plus près du pôle animal du côté des futurs quadrants C et B, que du côté des futurs quadrants D et A. Aucun plan de symétrie ne pourrait donc passer par l'axe animal-végétatif, mais on comprendrait aussi comment il se fait que *la plus grande partie du plasma incolore de CD passe en D*. Cette nouvelle répartition inégale des divers plasmes entre les quadrants C et D avait d'ailleurs déjà été observée par Carazzi (1905), qui remarque du gros vitellus (endoplasme) en D, mais surtout en C.

L'évolution du plasma incolore, qui passe en grande partie dans le blastomère CD et puis dans le quadrant D et dont la localisation semble bien en rapport avec l'inégalité des premières divisions de segmentation, nous incite à considérer ce plasma comme l'analogue d'un plasma polaire. Son rapport avec la morphogénèse est suggéré par le fait qu'il passe en grande partie dans le quadrant D, cellule-souche de la lignée mésoblastique. Moins dense que les plasmes polaires d'autres *Spiralia*, il ne passe pas complètement dans ce blastomère D. Ce dernier devrait ses potentialités mésoblastiques à une répartition du plasma incolore qui le favoriseraient quantitativement. Au lieu de 'repousser' le sillon de la cytotidérèse, comme le font les plasmes polaires denses, il l'"attirerait" au contraire. Un phénomène absolument comparable semble survenir à l'expulsion des micromères 1a et 1b; on observe en effet une déformation au pôle animal des blastomères A et B. Cette déformation, due à l'accumulation d'un nouveau plasma incolore, rappelle singulièrement celle qui touche l'œuf avant la première division de segmentation. C'est encore du côté de ce plasma fluide, qui n'a pas nécessairement la même composition que le 'plasma polaire' initial — et ici il apparaît clairement combien l'analyse cytochimique des phénomènes, qui nous occupent, s'impose — que vont se former les micromères.

Si cette interprétation correspondrait à la vérité, on trouverait dans le plasma incolore de l'œuf d'*Aplysie* 'la condensation de substances analogues à celle des plasmes polaires' dont la présence était suggérée à Pasteels par ses interventions expérimentales. On verrait également apparaître la corrélation entre les changements qui surviennent dans l'œuf à la fin de la maturation d'une part, la segmentation inégale et la morphogénèse d'autre part.

Dans le cadre des phénomènes initiaux du développement chez les *Spiralia*, l'*Aplysie* trouverait une place logique, quoique exceptionnelle. L'existence d'un

élément analogue à un plasme polaire serait en corrélation avec sa morphogénèse et avec le caractère inégal de sa segmentation. Ce 'plasme polaire' serait toutefois *animal* et plus fluide qu'habituellement chez les *Spiralia*. Par ce dernier caractère il 'attirerait' le sillon de segmentation, au lieu de le 'repousser' comme le font les plasmes polaires denses, ce qui expliquerait l'inversion de l'inégalité de la première division de segmentation en faveur du blastomère AB.

RÉSUMÉ

L'étude de la maturation et du début de la segmentation de l'œuf d'*Aplysia punctata* par la coloration vitale au bleu de Toluidine montre l'apparition d'un 'plasme incolore' et sa répartition inégale entre les divers blastomères. Il pourrait s'agir d'un élément analogue aux plasmes polaires d'autres *Spiralia*, en corrélation avec l'inégalité des divisions de segmentation et avec la morphogénèse.

SUMMARY

Study of the maturation and the beginning of segmentation of the egg of *Aplysia punctata* by vital staining with toluidine blue demonstrates the appearance of a 'colourless plasm' and its unequal distribution between the various blastomeres. It may be a component analogous to the pole plasms of other *Spiralia*, correlated with the inequality of the cleavage divisions and with morphogenesis.

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